The role of CD28 in T cell activation and the transcriptional regulation of the interleukin2-gene

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Award date:
1996

Awarding institution:
University of Bath

THE ROLE OF CD28 IN T CELL ACTIVATION AND THE TRANSCRIPTIONAL REGULATION OF THE INTERLEUKIN-2 GENE

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for the degree of PhD.

of the University of Bath 1996

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I dedicate this thesis to Mum, Dad and Ed without whose love, patience (and financial support !) I would not have had the will nor the means to undertake this PhD.

Thank you

There's a time, a place we've yet to know
There's a sea we've yet to sail
And though we saw the mountain long ago
It's a peak we've yet to scale

ACKNOWLEDGEMENTS

There are so many people who have provided endless support, information and time during the duration of this PhD that the list is almost as long as the thesis. I wish to thank them all most gratefully and hope that someday I may be of use in return. There are however, certain individuals without whom I would not have succeeded in this PhD and whom I would like to thank personally.

To my supervisor, Dr. David Sansom who has suffered endless disappointing results and frequent interuptions I would like to say thank you for all your help, suggestions and unfailing support throughout the three years.

To Dr. Nick Hall, thank you for your confidence in my research ability when offering me the PhD position and your continued support during my time at B.I.R.D.

To Dr. Andrew Lennard at the Yamanouchi Research Institute in Oxford for all his help with the EMSA and footprinting assays.

To Cor Verweij and his group at the Central Bloodbank Laboratories in Amsterdam for their help with the EMSA

A big thank you to all my friends at B.I.R.D. for all their help and for such an enjoyable three years. I miss you all already!

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ABBREVIATIONS

APC	Antigen Presenting Cell
ARAM	Antigen Recognition Activation Motif
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol Acetyltransferase
CD	Cluster of Differentiation
CD28RC	CD28 Response Complex
cDNA	complementary Deoxyribonucleic Acid
CHO cell	Chinese Hamster Ovary cell
CIP	Calf Intestinal Phosphatase
CMV	Cytomegalo Virus
CsA	Cyclosporin A
CTL	Cytotoxic Lymphocyte
CTLA-4	Cytotoxic Lymphocyte-associated Antigen-4
DAG	Diacylglycerol
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic Acid
EAE	Experimental Allergic Encephalomyelitis
EGF	Epidermal Growth Factor
EMSA	Electomobility Shift Assay
ERK	Extracellular signal- regulated kinase
FACS	Fluorescent Activated Cell Sorter
FCS	Foetal Calf Serum
FDA	Fluorescein Diacetate
FITC	Fluorescein Isothiocyanate
GDP	Guanine Diphosphate
GM-CSF	Granulocyte/ Macrophage- Colony Stimulating Factor
GST	Glutathione S-Transferase
GTP	Guanine Triphosphate
HLA	Human Leukocyte Antigen
IFN	Interferon
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-2R	Interleukin-2 Receptor
IP_3	Inositol Triphosphate
JNK	c-Jun amino terminal Kinase
kbp	kilo base pairs

kDa	kiloDalton
LFA-3	Leukocyte Function-associated Antigen-3
mAb	monoclonal antibody
MAPK	Mitogen Activated Protein Kinase
MEKK	MAPK and ERK Kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
mRNA	messenger Ribosomal Nucleic Acid
NFAT	Nuclear Factor of Activated T cells
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PI3 Kinase	Phosphoinositol-3- Kinase
PIP_2	Phosphoinositol Bis Phosphate
PKA/B/C	Protein Kinase A/B/C
PLC	Phospholipase C
PMA	Phorbol 12-Myristate 13-Acetate
PNK	Poly Nucleotide Kinase
PTK	Protein Tyrosine Kinase
ROI	Reactive Oxygen Intermediate
SDS-PAGE	Sodium Dodecyl Sulphate- polyacrylamide gel electrophoresis
SEA/ SEB	Staphylococcal Enterotoxin A/ B
SH2/ 3	Src Homology domain 2/ 3
SOS	Son Of Sevenless
TCF	Ternary Complex factor
TCR	T Cell Receptor
TNF	Tumour Necrosis Factor
TPA	12-O-tetradecanoylphorbol-13-acetate
ZAP	Zeta associated protein

ABSTRACT

Despite the realisation of the importance of CD28 in T cell activation very little is currently known concerning the nature of the costimulatory signals. Research has been hampered by the requirement for TCR engagement to enable observation of a costimulatory effect, making it difficult to assess the relative contribution of CD28 signals as distinct from those of the TCR. The observation that activated human T cell blasts could be stimulated via the CD28 surface molecule in the absence of antigenic challenge thus provided a very useful model for the investigation of costimulatory signals in isolation. Utilising SEA-responsive T cell blasts the effects of CD28 mediated costimulation on IL-2 gene transcription and the signalling pathways involved, were investigated.

Whilst stimulation of the quiesced T cell blasts with DR4/B7-SEA transfectants induced full activation and IL-2 production the addition of CHO-B7 cells alone resulted in time dependent proliferation but no observable IL-2 output. These latter responses were shown to be sensitive to inhibition by a number of agents including CsA, rapamycin, and the PKC inhibitor Ro 31-8220. Furthermore whilst the acidic sphingomyelinase inhibitor, chloroquine completely inhibited all B7 induced proliferation, the PI3 Kinase inhibitor, wortmannin was found to have limited effects indicating that PI3 Kinase was not a major mediator of the B7-induced proliferation.

A study of transcription factor induction following B7 stimulation revealed the existence of two CD28 regulated factors, AP-1 and NFκB; the absence of NFAT induction correlating with the lack of IL-2 production. Preincubation of the cells with chloroquine inhibited the induction of both factors whilst wortmannin only affected AP-1 expression. This suggests that whilst CD28 may signal via both pathways, the activation of acidic sphingomyelinase appears to be of greater importance in these activated cells.

CHAPTER ONE - INTRODUCTION

1.0 THE IMMUNE SYSTEM

The highly complex immune system has evolved as a protection against invading pathogens. It comprises a number of different cell types with specific functions in the mediation of an immune response and it is the interaction between and the controlled activation of these cells which confers efficiency and specificity to an immune response. One of the major mechanisms by which immune protection is achieved involves the processing of foreign antigens and their presentation to thymus-derived lymphocytes (T cells) by APCs. This event represents the initiation of an immune reaction involving both cell-mediated and antibody-mediated responses and results in eradication of the foreign antigen and development of immune memory to aid subsequent challenge by antigen.

Several different subsets of T cells have been described including helper (Th) and cytotoxic (CTL) T cells, characterised by their expression of surface molecules and the effects induced following their activation. Whilst $CD8^+$ CTL cells respond to antigens presented by class I Major Histocompatibility Complex (MHC) molecules and generally elicit a cytotoxic response following activation, MHC class II reactive $CD4^+$ Th cells respond to antigen by cytokine production and activation of bone marrow derived lymphocytes (B cells). The $CD4^+$ subset, comprising approximately 60% of peripheral blood T lymphocytes, has been shown to play a major role in the specific recognition and response to foreign antigens.

1.0.1 ANTIGEN PRESENTING CELLS AND PEPTIDE PRESENTATION

Resting $CD4^+$ peripheral blood T cells whether they are naive ($CD45RO^-$) or memory cells ($CD45RO^+$) require two signals for full activation (review Schwartz, 1990). The first signal (signal 1) is delivered via the TCR and *in vivo* is usually triggered following the ligation of the TCR by an antigenic peptide bound to a MHC class II molecule on an antigen presenting cell (APC). Several different cell types can act as APCs including monocytes, activated B lymphocytes and dendritic cells (Yokochi et al., 1982; Freedman et al., 1991; Young et al., 1992; Hart et al., 1993). All are positive for the Human Leukocyte antigen (HLA-DR) alleles and have the ability to present antigen in the context of other required adhesion and costimulatory molecules. *In vivo* the presented antigen is usually a peptide derived from a larger antigenic protein. Following invasion of the body by the foreign antigen, the proteins are phagocytosed by macrophage-like cells, broken down into smaller peptide fragments, incorporated into

the peptide binding grooves of the MHC molecules and then presented on the surface of the APC. The immunogenicity of each peptide fragment is dependent upon its ability to bind an autologous MHC molecule as the TCR recognises the whole complex and not just the peptide.

1.0.2 THE TCR AND THE T CELL REPERTOIRE

The TCR is a glycoprotein heterodimer comprising αβ or γδ chains (review Eckels, 1990); the former being expressed in 95% of peripheral T cells. The structure of these α and β chains closely resembles that of an antibody in that both possess variable (V), joining (J) and constant (C) regions, however, the β chain also contains a diversity domain (D) (Davis and Bjorkman, 1988). Within the V/Jα and the VDJβ domains lie three hypervariable regions called the Complementarity Determining Regions (CDRs) and it is these which confer the recognition specificity onto the TCR. Studies of the interactions occurring between the TCR/ peptide- MHC complex are still in progress but it appears that the CDR1 and CDR2 may contact the MHC molecule whilst the CDR3 interacts with the peptide (Davis and Bjorkman, 1988). As there are several existing alleles for each V, D and J domain the complex mechanism of gene rearrangement and recombination of the domains generates extremely large numbers of T cells with diverse TCR specificities. However, whilst this enables an individual to respond to an enormous range of antigenic peptides, at the same time, the generated T cell repertoire includes a large number of potetially autoreactive T cells.

1.0.3 THYMIC SELECTION

An autoreactive T cell is one which recognises an self antigen in the context of a self MHC molecule and is therefore potentially harmful to the body. In order to prevent self-reactivity, developing T lymphocytes undergo a selection procedure in the thymus to remove self or non responsive T cells. Whilst negative selection removes T cells which do not recognise autologous MHC, the removal of self-reactive T cells is known as positive selection or clonal deletion (Kappler and Marrack, 1987) and results in the death of T cells which respond to self antigen-MHC presented by thymic APCs. Although very little of this process is currently understood it does appear to be the major mechanism for the removal of autoreactive T cells and occurs during development prior to the release of the thymocytes into the periphery.

1.0.4 TOLERANCE

There is evidence indicating that some self reactive cells escape selection and procede to the periphery. However, it appears that their activation is usually suppressed by as

yet little understood mechanisms, broadly classified as peripheral tolerance. This was originally thought to involve a third defined subset of T cells known as suppressor cells although the existence of such cells has been difficult to demonstrate. An alternative mechanism proposed for the functional suppression of such cells is clonal anergy which is discussed in more detail below. Since potentially autoreactive cells can be detected in the peripheral blood of normal healthy individuals an active suppression process must be occurring. Unfortunately occasionally a lapse in peripheral tolerance or a direct activation of self-reactive T cells occurs which can result in the onset of autoimmune disease. Rheumatoid Arthritis is a common example during which an immune response is mounted against the joints resulting in a very painful and crippling disease.

The reason for a failure in peripheral tolerance is not known but it is unlikely that there is a single cause. Since two signals are required for T cell activation the failure could reside with either the antigen or the costimulatory molecule. Epitope mimickry whereby the self antigen/ MHC complex resembles an antigenic peptide has been proposed as one possible mechanism whilst tissue injury can result in the exposure of self antigens not encountered and selected against in the thymus. Alternatively self-reactive T cells may be activated if they encounter high concentrations of self antigen in the presence of aberrant costimulatory molecule expression. Numerous studies have been carried out to attempt to establish the trigger factors and although some progress has been made with the identification of susceptibility markers the discordancy of autoimmune disease in families and twins argues against a purely genetic aetiology and implies the additional involvement of an environmental trigger (Zanelli et al., 1995). Research has shown that different MHC class II alleles can present an antigen in different ways which may result in an alteration of the immunogenicity of the peptide. This has been demonstrated in patients with R.A who, compared to the general population, have a predominance of HLA-DR4Dw4 and HLA-DR1Dw1 alleles (Zanelli et al., 1995). This implies that such alleles may be more efficient at presenting self joint peptides and these are now regarded as susceptibility markers for the disease. In contrast other alleles have been demonstrated to have protective effects including HLA-DR2, HLA-DR7 and HLA-DR8 (Zanelli et al., 1995). However, these genetic markers are not absolute and as the identity of the trigger factors are still not known further research is required before effective treatments of such autoimmune diseases will be possible.

1.0.5 ANERGY AND APOPTOSIS

Activation of the TCR in the absence of a second or costimulatory signal results in the cell becoming hyporesponsive or anergised (review Schwartz, 1993) which has been observed in a number of *in vitro* models. Jenkins et al (1990) characterised the anergic

state as a failure of αCD3-stimulated resting T cells to respond to further stimulation and a lack of IL-2 production, whilst Lamb et al (1983) demonstrated similar effects in peptide-stimulated T cell clones. Interestingly the stimulation of T cells using fixed APCs also induces anergy, presumably due to the inability of these cells to upregulate the costimulatory molecules following TCR/MHC ligation (Jenkins and Schwartz, 1987). Anergy can be reversed by incubation of the cells with interleukin-2 (IL-2) (Beverly et al., 1992) and is thought to be a protective mechanism to prevent T cell activation in inappropriate situations. Clonal anergy has been proposed to be the major mechanism involved in the maintenance of peripheral tolerance, induced in autoreactive T cells when encountering self antigen in the absence of a costimulatory molecule. The induction of anergy may also underlie the mechanism of natural and induced tolerance since *in vivo* challenge with antigen alone has been shown to result in a long term hyporesponsiveness to the antigen. This phenomena is currently being investigated as a possible treatment for allergy (Yssel et al., 1994).

A second mechanism for downregulation of a cell is apoptosis or programmed cell death (PCD) (MacDonald and Lees, 1990; Cohen et al., 1992). This is an active process, distinct from normal cell necrosis, during which the cells receive specific stimuli causing the condensation of DNA, chromosomal degradation and budding of the nuclei followed by a disintegration of the cell. Several molecules have now been identified which can positively (e.g Fas, TNF, CTLA-4) (Itoh et al., 1991; Alderson et al., 1995; Dhein et al., 1995; Smith et al., 1994; Schultz-Osthoff et al., 1994; Gribben et al., 1995) or negatively (bcl-2) (Boise et al., 1993b; Bissonette et al., 1993) regulate the apoptotic process. This is a rapidly expanding field which is too complex for a detailed discussion here although several recent observations indicate that the factors regulating T cell activation and apoptosis show considerable overlap. TCR induction alone has been reported to upregulate Fas ligand on the surface of T cells whilst CTLA-4 is bound by the CD28 ligands B7-1 and B7-2 and appears to activate similar signalling pathways. Thus the outcome of T cell stimulation seems to be far more complex than which surface molecules are ligated and may involve interactions between several intracellular signalling pathways.

1.1 COSTIMULATION

The required second signal necessary for full T cell activation is provided by a costimulatory molecule. As demonstrated by experimental data, candidate molecules for such a costimulatory task must satisfy a number of specified criteria. These were summarised by Jenkins et al (1988) as: a) the ability to provide costimulation from a cell not providing signal 1 b) the ability to provide a signal which can synergise with

CD3 stimulation resulting in proliferation and c) the ability to generate a signal which is time dependent in its ability to induce proliferation. One candidate costimulatory molecule meeting these criteria is CD28, a 44kDa homodimeric surface molecule expressed constitutively on 95% of $CD4^+$ T cells (June et al., 1990; Turka et al., 1990). Engagement of CD28 by its ligand B7 has been proposed as the main costimulatory signal in the activation of resting T cells (Linsley et al., 1991a; Gimmi et al., 1991) although the discovery of secondary ligands for both CD28 (B7-2) (Freeman et al., 1993b; Azuma et al., 1993) and B7 (CTLA-4) (Linsley et al., 1991b) has now made the situation more complex. A diagram summarising the interaction of these different molecules is shown in figure 1.0.

The criteria specified above discount adhesion molecules as costimulators. Although these molecules appear to be able to synergise with CD3 stimulation to induce proliferation, they are only effective on the same cell as the MHC class II molecule therefore the purpose of many of these adhesion molecules may simply be to bring the two cells into close proximity for the stimulatory interactions to occur. Varying abilities of B7 and LFA3 to costimulate anti-CD3 - induced responses were detected by Sansom et al (1993) depending upon the origin of signal 1. Using purified T cells only B7 could costimulate an anti-CD3 signal whilst both B7 and LFA3 could costimulate the lectin, phytohaemagglutinin (PHA), with an additive signal observed if given in combination. Stimulation with the superantigen Staphylococcal enterotoxin B (SEB) required the presence of a class II MHC molecule and whilst both B7 and LFA3 could costimulate this signal if on the same cell, only B7 was operative from a third party cell. Such findings indicate a complex interaction of different signalling pathways and a requirement for synergy to induce a full activation response. Lucas et al (1995) demonstrated that naive T cells, deficient in CD28, could be induced to proliferate using high concentrations of antigen alone. The response was vigorous but short lived and proliferation ceased completely after 48 hours, furthermore, no IL-2 was produced by the cells during the stimulation. Addition of a B7 costimulation to $CD28^+$ cells decreased the concentration of antigen required and resulted in a prolonged proliferation and IL-2 production.

1.1.1 B7-1

The first identified ligand or counter receptor for CD28, B7/ BB-1 (CD80) was a 30 kDa, type 1 transmembrane glycoprotein. This is a member of the immunoglobulin superfamily, having two immunoglobulin (Ig) like extracellular domains consisting of a single ' V' domain and a single Ig constant domain (Freeman et al., 1989). B7-1 was first described as an inducible B cell antigen whose expression could be induced by a number of factors including class II MHC ligation with TCR, cAMP and Interferon γ

(IFNγ). It has been detected on many types of APCs and also on repeatedly activated T cells (Sansom and Hall, 1993) but in contrast to the constitutive expression of CD28 on resting T cells, B7 is only expressed on activated APCs. Increased expression of B7 after MHC class II/ TCR binding implies there may be functional signal transduction through the MHC molecule as well as via the TCR (Nabavi et al., 1992). Upregulation of B7 was also observed in cultured dendritic cells which had not been directly stimulated but may have responded to cytokines produced by other cell types (Hart et al., 1993). An example of this was observed on stimulation of B cells with IL-2 and IL-4 which both induced B7-1 expression whilst IL-7 has been shown to upregulate B7 levels on activated T cells (review June et al., 1994).

The requirements for B7 in T cell activation have been investigated by numerous groups (Gimmi et al., 1991; Harding et al., 1992; Linsley et al., 1991a) and have involved the use of stimulatory and blocking monoclonal antibodies to CD28 as well as CTLA-4 Ig fusion molecules. The CTLA-4 Ig chimeric constructs comprise the extracellular domain of the second B7 ligand, CTLA-4, with the constant region of the IgG1 heavy chain. This forms a non- stimulatory, soluble complex with a high affinity for the B7 molecules which will block interactions between B7 and CD28. Addition of this complex during alloantigenic challenge has been shown to result in a hyporesponsive state or anergy (Linsley et al., 1992b). Furthermore long term survival of both allogeneic and xenogeneic grafts have been observed in costimulatory deficient transplants (Lenschow et al., 1992) hence B7-blocking molecules may have an important role to play in immunotherapy. Conversely, transfection of tumours to induce overexpression of B7 has been found to decrease tumour growth and promote recovery (Townsend and Allison, 1993), however, the mechanism behind the latter appears to be more dependent upon cytolytic $CD8^+$ T cells. Although activated by the tumours bearing antigen on class I MHC, the lack of costimulation makes the $CD8^+$ cells dependent upon $CD4^+$ help for IL-2 and proliferation. Transfection of B7 into the tumour cells provides the missing costimulation resulting in $CD8^+$ cell IL-2 production and clonal expansion followed by cytolysis of the tumour cells. Interestingly further challenge with B7-negative tumour cells also resulted in cytolysis due to the presence of the preactivated $CD8^+$ cells and cross reactivity of the tumour antigens suggesting tumour immunisation. Thus B7-1 would appear to be an extremely important regulator of the T cell activation process and may therefore be a major target for manipulation of the immune system.

1.1.2 B7-2

The second CD28 ligand to be described, B7-2 (CD86) is a 70kDa glycoprotein, which although a member of the same immunoglobulin family, shows limited sequence

homology to B7-1 (Azuma et al., 1993). In contrast to B7-1, B7-2 is expressed constitutively on the surface of resting monocytes and dendritic cells and on activated lymphocytes and therefore may be the first ligand encountered by CD28 during the activation process.

Studies comparing the effects mediated by the two B7 molecules have produced conflicting results. Lanier et al (1995) could find no differences in the T cell responses to B7-1 or B7-2 with regards to their ability to costimulate proliferation, cytokine production and the generation of cytotoxic lymphocytes (CTL). However, two groups have reported that B7-1 and B7-2 differentially activate T helper cell (Th) subsets. Although equal stimulators of IL-2, IL-2 receptor (IL-2R) and IFNγ, B7-2 is capable of inducing much higher levels of IL-4, especially in naive cells, which has the effect of pushing the cells towards a Th2 phenotype (Freeman et al., 1995). Kuchroo et al (1995) found that administration of B7-1 or B7-2 specific antibodies to an EAE model could also influence the outcome of the disease. Whilst blocking B7-1 decreased the disease incidence, blocking B7-2 increased the disease severity. This is consistent with B7-2 driving the protective Th2 phenotype and B7-1 the inflammatory mediating Th-1 subset. However, during an allergic reaction it is the Th2 subset which mediates the hypersensitivity response (Yssel et al., 1994) whilst the Th1 subset is protective. Further research is required to confirm these differences but if correct the information may be able to be utilised in the design of more specific therapeutic treatments for the control of autoimmune disease.

1.1.3 CD28

The human CD28 gene is arranged into 4 exons resulting in four different mRNA transcripts depending upon the gene splicing. The first pair, 3.7 and 3.5kbp have poly adenylation extensions compared to the second pair, 1.5 and 1.3kbp. The difference within the pairs represents the extracellular domain deletion encoded for in exon 2 (Lee et al., 1990). The CD28 gene possesses an AP-1 transcription factor regulatory binding site in the 5' untranslated region which explains its inducibility by phorbol esters, however, under more physiological conditions the CD28 gene is regulated by TCR-derived signals and B7 ligation. CD28 levels rise following T cell activation through the TCR, presumably to increase the cells ability to receive costimulation. Following B7 ligation of CD28 there is a transient decrease in CD28 mRNA lasting 4-24 hours accompanied by a slower but more prolonged decrease in surface expression of the molecule (Linsley et al., 1993).

It has long been known that CD28 exerts one of its costimulatory functions via stimulation of IL-2 production. This is achieved in two ways; the first by a direct effect

on IL-2 gene transcription and the second by a stabilisation of the IL-2 mRNA. Little is known about the mechanism controlling the latter although it is believed to involve a CD28-mediated inhibition of mRNA degradation which relies on an AU rich region at the 3' terminus of the mRNA (Lindsten et al., 1989b). Details of the effects of CD28 on gene transcription will be discussed further.

CD28 also appears to have a direct effect on the expression of the IL-2R; a trimeric complex comprising the α,β and γ subunits. Different affinity receptors have been identified and associated with different combinations of the subunits. The high affinity receptor comprises all three subunits whilst the intermediate, βγ and the low affinity receptor only α. The latter appears to be a sink for excess IL-2 since the β and γ subunits are required for receptor internalisation and signalling (reviewed by Taniguchi and Minami, 1993). The β subunit is also shared with the IL-15 receptor (Giri et al., 1994) and may explain similarities in the properties of the two cytokines. Unlike the β subunit which is expressed constitutively, the α subunit is only expressed after stimulation. This upregulation can be induced by CD28 alone but is considerably enhanced in level and duration by the concomitant stimulation of CD2 (Cerdan et al., 1992). This combination of signals also stimulates increased IL-2Rβ expression via mechanisms which again seem to involve gene transcription and mRNA stabilisation. However, the IL-2Rβ mRNA does not bear the usual AU rich sequence required for CD28 mediated stabilisation so how this is achieved is not yet known. The increase in IL-2Rβ occurs slightly later than the upregulation of IL-2Rα but can be explained by the differences in the critical regulatory transcription sites. IL-2Rα transcription depends upon activation of an NFκB motif within its promoter region to increase gene transcription (Arima et al., 1992) whereas the IL-2Rβ subunit is dependent upon an Ets binding site (Lin et al., 1993). The significance of these differences have yet to be realised.

As well as an effect on IL-2 and IL-2R, CD28 can also regulate the levels of numerous other cytokines including IL-3, IL-5, IL-10, GM-CSF, TNFα, IFNγ and IL-4. The increase in IL-4 production following CD28 activation is moderate compared to its effects on IL-2 but appears to be more pronounced in cells with a low IL-2 output. Interestingly the production of IL-4 appeared to be mediated via IL-2 as an inhibitory mAb to IL-2 and IL-2R decreased the observed levels of IL-4. This also appeared to be true for the other reported CD28-regulated cytokines IL-5, IL-10 and IFN-γ (Kuiper et al., 1994b). However, CD28-responsive elements (CD28RE) have been identified in the gene promoter regions of IL-3, GM-CSF and IFN-γ (Fraser and Weiss, 1992) indicating a direct effect on the gene. Further research is therefore awaited to clarify these effects.

1.1.4 CTLA-4

CTLA-4 was discovered following a search for T cell surface antigens which were involved in cytotoxicity (Brunet et al., 1987) during which, closer examination of this molecule revealed significant homology to the costimulatory molecule CD28. Both are members of the immunoglobulin supergene family with a single extracellular ' V' - like domain and map to the same region of the human genome, chromosome 2q33. Their similarities in structure, sequence homology and gene location point to an evolutionary gene duplication (Harper et al., 1991).

Until very recently the knowledge of the properties of CTLA-4 was little more than a series of apparently contradictory, isolated observations. Unlike CD28, CTLA-4 expression levels on resting T cells are extremely low but can be increased by activation of the cells with a combination of TCR and CD28 mediated signals. The increase in expression is slow and only becomes maximal after 48-72 hours. Even at this stage CTLA-4 expression is only 2-3% of CD28 expression (Linsley et al., 1992a). This may appear so insignificant as to be irrelevant until it is realised that the avidity of CTLA-4 for the CD28 ligands B7-1 and B7-2 is 20-100 fold higher than for CD28 itself (Linsley et al., 1991b). It may therefore be unnecessary for the expression levels of CTLA-4 to be that high in order to achieve a maximal response.

The effects of CTLA-4 stimulation are still a matter of conjecture. *In vitro* studies have indicated both stimulatory and inhibitory effects for CTLA-4 activation. Krummel and Allison (1995) found that in the presence of immobilised anti-CD3, soluble CTLA-4 mAb synergised with and potentiated the proliferative effect of anti CD28. However, if the CTLA-4 and CD28 mAbs were also immobilised proliferation and IL-2 production was inhibited. Several reports have indicated that unlike CD28, stimulation of CTLA-4 leads to apoptosis (Gribben et al., 1995) and in further support of this are two recently published studies concerning the fatal lymphoproliferative disorders observed in a strain of CTLA-4 deficient mice (Waterhouse et al., 1995; Tivol et al., 1995). Thus it now appears evident that CTLA-4 has a negative regulatory role in the control of T cell activation and proliferation, however, further detailed studies will be necessary to elucidate the differences in the signalling pathways of CD28 and CTLA-4.

Like CD28 CTLA-4 has been shown to bind both B7-1 and B7-2 and does not appear to significantly differentiate between the two in terms of affinity. A rather surprising discovery was the higher affinity of CTLA-4 for the B7 ligands, compared to that of CD28, since it had been thought that CD28, due to its high level of expression, would play the major role. However, when considering the constitutive expression and inducibility of the ligand pairs it would appear that CD28 may be the primary ligand for

B7-2 whilst the CTLA-4 / B7-1 interaction could occur 48-72 hours after activation when the surface expression of these inducible molecules is maximal. Due to its delayed expression it appears the role of CTLA-4 could be secondary to the initial CD28 costimulation and may cause the termination of the proliferative response.

Many of the CD28/ B7-1 costimulation studies carried out so far have involved the use of gene knockout or transgenic mice. These provide an invaluable tool for the study of the importance of these molecules and the roles they play in T cell activation. Whilst B7-1 deficient mice appeared, developed and reacted normally to antigenic challenge (Freeman et al., 1993a), CD28 deficient mice showed a considerably impaired immune response (Shahinian et al., 1993). This provides further evidence indicating that CD28 can be activated by alternative ligands other than B7-1 whilst the presence of a functional CD28 receptor is an absolute requirement of an efficient immune system and gives credence to the theory that the CD28/ B7-2 interaction may be the primary and most essential costimulus in the activation process.

1.2 SIGNAL TRANSDUCTION

To obtain a greater appreciation of these activation molecules and how they function it is necessary to understand the mechanisms by which surface receptor stimulation effects a change in the activation state of the cell. Downstream signalling from surface receptors can be organised into a series of linear signalling pathways which ultimately converge to affect gene transcription. However, several key proteins have been identified including the GTP-binding protein, ras and the mitogen activated family of serine /threonine kinases (MAPK) which appear to impinge upon a number of different pathways and may act as junction switches to control the final outcome of the surface receptor stimulations.

1.2.1 TCR-CD3 SIGNALLING

Interactions between the TCR/ CD3 complex on the T cell and the MHC class II molecule initiate a complex series of events which result in activation of the T cell, lymphokine production and proliferation. Gradually the mechanisms of this signalling cascade have been unravelled although with the discovery of each new protein kinase or phospholipase the overall picture becomes an ever more complex network of interacting and overlapping pathways. However, the physiological significance of these different pathways and the mechanisms by which they are triggered remains to be elucidated. Stimulation of the TCR/ CD3 complex in conjunction with the CD4 molecule leads to activation of phospholipase Cγ1 (PLC) resulting in the production of inositol triphosphate (IP_3) and diacylglycerol (DAG). The former promotes the release of

calcium from intracellular stores whilst the latter activates PKC. Although the pathways diverge at this point they reconverge downstream in the activation of the transcription factor NFAT, a DNA binding complex comprising a PKC-induced nuclear subunit and a constitutive cytoplasmic subunit which is translocated to the nucleus following calcium stimulation. This general overview of TCR signalling is summarised in figure 1.1.

Research undertaken recently has shown this description of TCR signalling to be a considerable oversimplification and although the picture is not yet complete several gaps in our knowledge have been filled. The first discovery was that the TCR/ CD3 complex actually comprises seven different subunits. The majority of $CD4^+$ helper T cells express the $\alpha\beta$ heterodimeric TCR, although the existence of $\gamma\delta$ populations are known. The $\alpha\beta$ molecule is responsible for the specific recognition of and interaction with the antigenic peptide in the MHC class II groove. The cytoplasmic tails of these $\alpha\beta$ chains, however, are extremely short and do not participate in signal transduction; a job which falls to the coassociated CD3 invariant chains. Five different chains have been described γ, δ, ε, ζ and ν which associate non covalently with each other and with the $\alpha\beta$ TCR. These chains have a dual purpose being required for the assembly and surface expression of the TCR as well as transducing the signal initiated by antigen recognition. One common feature of the cytoplasmic tails of these molecules is the presence of a highly conserved signalling motif. Originally known as the Reth motif, it is now more commonly referred to as the TAM (tyrosine - activated motif) or the ARAM (antigen recognition activation motif) sequence (Reth, 1989). The sequence consists of paired tyrosine and leucine or isoleucine residues in the form Y-XX-L-X_{7-8}-Y-XXL. Whilst the majority of ARAM containing proteins carry one copy of the sequence the CD3 ζ and ν chains carry multiple copies, three and two respectively (Sancho et al., 1993). Coupled with the fact that the ζ chain can function in a number of homo and heterodimeric forms (ζ–ζ, ζ–~ and ζ–γ) the indication is that this may operate as the main signal transducing molecule. Studies carried out in transformed cell lines using chimeric CD8α /CD3 ζ constructs showed that antibody induced ligation of CD3 ζ was sufficient to induce T cell activation as measured by tyrosine phosphorylation, calcium flux and IL-2 production (Koysau et al., 1994). However, Brocker and Karjalainen (1995) later proved that this was not the case for resting T cells which required full receptor stimulation and costimulation to induce activation. This group also showed, however, that previously activated T cells behaved in a similar fashion to transformed cells indicating that once the activation has been initiated fewer signals are required to maintain the activated state. Further studies of the function of the ARAM motif indicated a requirement for phosphorylation on the tyrosine residues prior to the association with the SH2 domains of the Syk- family protein kinase ZAP-70 (ζ

associated protein) (Chan et al., 1992). This phosphorylation is thought to be carried out by the src family kinases lck and fyn (Chan et al., 1995).

1.2.2 PROTEIN TYROSINE KINASES

Protein tyrosine kinases can be subdivided into two families; the membrane associated src family comprising p56 lck and p59 fyn (reviewed (Rudd et al., 1994)) and the cytoplasmic syk family comprising syk and ZAP (Chan et al., 1994). ZAP-70 is required for T cell development and TCR function and can associate with any tyrosine phosphorylated ARAM containing protein via its two SH2 domains although the association with CD3ζ is best described. ZAP-70 itself undergoes phosphorylation on tyrosine 493 following TCR activation resulting in catalytic enzymatic activity and it is proposed that this phosphorylation event is undertaken by a src family kinase, probably p56 lck (Chan et al., 1995). The src family members lck and fyn contain both SH2 and SH3 domains and membrane-associate via cotranslational myristylation and post translational palmitylation (Chan et al., 1995).

The associations occurring between these various tyrosine kinases appear rather complex. The CD3ζ chains require phosphorylation before they can bind ZAP which itself requires phosphorylation in order to bind lck which has been proposed as one of the main candidates for the original phosphorylations of CD3ζ. Another candidate p59 fyn has been found to associate with the CD3 chains in resting cells via the ARAM sequences, however, its binding requirements appear to be less specific than those observed for ZAP. Single mutations within the ARAM motif had little effect upon fyn binding but completely abrogated ZAP associations. Furthermore single base pair deletions disrupted ZAP binding but not that of fyn (Timson-Gauen et al., 1994). One possible explanation is that fyn association is more dependent upon the interaction between its associated fatty acids and the secondary structure of the ARAM motif than on the binding of specific amino acid residues within the motif.

Lck binds to the CD4 molecule whilst fyn is TCR associated but both have been shown to associate with a presently unidentified 120kDa protein in unstimulated Jurkat cells via SH3 domain binding (Reedquist et al., 1994). Phosphorylation of this 120kDa molecule is apparently one of the first signalling events, occurring within 10 seconds of CD3 stimulation and peaking at 30 seconds. It is thought that the phosphorylated protein may then interact with the SH2 domains of the PTKs in order to transduce further signals. These interactions between the TCR/ CD3 complex and the associating PTKs are shown in figure 1.2 along with the structure of the PTK binding domains.

1.2.3 THE ROLE OF PKC

Currently the exact nature of the signals transduced by the PTKs is not known and although it is possible to identify downstream targets of TCR signalling the links between them are still somewhat uncertain. Much of the signalling data has been confused by studies carried out in a variety of T cell types. It is clear that the operational signalling pathways in immortal transformed cell lines such as Jurkats vary considerably from those observed in T lymphoblasts which again may vary from resting peripheral T lymphocytes. Such an example is the effect of PKC activation on the TCR induced calcium signal as investigated by Cantrell et al (1989). Following TCR ligation intracellular calcium levels rise by two mechanisms. The first involves a release of calcium from intracellular stores by IP_3, a metabolite of PIP_2 hydrolysis mediated by PLC, whilst the second mechanism is via an influx of calcium from outside the cell through membrane channels. In Jurkat cells PLC activation appears to be inhibited by PKC whereas in T lymphoblasts PKC activation has no direct effect on IP_3 levels (Ward and Cantrell, 1990). The reason for this difference is still unknown although studies of enzyme heterogeneity have indicated differences in PKC and PLC isotypes in the two cell types. Currently at least eleven different PKC isotypes have been identified (Hug and Sarre, 1993) which can be subdivided into three groups. The first comprising α, β_1, β_2 and γ are calcium dependent and can be activated by phorbol esters. The second group δ , ε, ν and τ are calcium independent but still respond to phorbol esters whilst the final three PKC ζ, λ and ι are calcium independent and phorbol ester insensitive. Differences between members of the same group have yet to be elucidated. The main isotype in Jurkat cells appears to be PKCα whilst the β form is more predominant in T lymphoblasts. The significance of this difference has not yet been elucidated although considering the involvement of PKC enzymes in regulating intracellular pathways it may account for a number of the abnormal responses observed in most immortal cell lines in response to phorbol ester stimulation.

1.2.4 THE MAPK CASCADE

It is not yet known what role PKC has in the activation of the downstream signalling molecule ras which appers to be at the start of a number of protein kinase cascades. At present the literature is still confusing with various studies claiming PKC to be upstream and/ or downstream of ras signalling (Williams et al., 1995).

Ras is a 21kDa GTP binding protein which is responsible for the activation of the MEKK, raf-1, by mediating its translocation from the cytoplasm to the plasma membrane (Leevers et al., 1994). Ras itself is activated by SOS, a nucleotide exchange factor which converts the inactive GDP bound ras to an active GTP bound form which

can then bind the amino terminal regulatory domain of raf-1 (Daum et al., 1994). Inactive ras is regenerated by GAP (Kazlauskas, 1994). At the membrane the ras bound raf-1 undergoes activation by an as yet unknown mechanism involving phosphorylation on a number of serine/ threonine and tyrosine residues. One suggested candidate for the serine/ threonine phosphorylations is PKCα (Daum et al., 1994) whilst the src kinases may be responsible for the tyrosine phosphorylation. Ras activation of raf-1 begins the serine/ threonine protein kinase cascade consisting of a series of phosphorylation reactions each activating the next downstream kinase. The target of raf is MEK or MAPKK (Jelinek et al., 1994), a dual specificity kinase which can phosphorylate both threonine and tyrosine residues of MAPK which appears to be an important molecule in the signalling pathway. Two members of the MAPK family which have been isolated are named ERK-1 and ERK-2 (review Ruderman, 1993). Their targets are numerous but all appear to have the role of releasing the cell from cycle arrest. They include the phosphorylation of the regulatory TCF/ Elk-1 component of the c-fos gene promoter (Marais et al., 1993) and phosphorylation of the serine/ threonine kinase pp90RSK S6 kinase (Erikson, 1991), not to be confused with the p70 S6 kinase to be discussed later. A current overview of the TCR-associated signalling pathways is given in figure 1.3.

1.2.5 THE JNK SIGNALLING PATHWAY

In addition to c-fos activation the MAP kinases have also been implicated in the phosphorylation of c-jun although this was shown not to be mediated by ERK but by other members of the MAPK family, the c- jun amino terminal kinases , JNK1 and 2 (Hibi et al., 1993). A study by Su et al (1994) showed that whilst the MAP kinases could be activated via PMA or TCR signals alone, JNK activation required two signals. These could be provided by PMA and ionomycin or physiologically following TCR and CD28 ligation. This was confirmed by a sensitivity of JNK activation to CsA and that PMA alone or constitutive ras only resulted in a partial induction of JNK. This latter observation of a single stimulus producing partial induction, however, was made in Jurkat cells and may not be representative of normal T cells. The requirement for two signals to induce full activation of the enzyme and the induction observed in the presence of TCR and CD28 ligation indicates that JNK may have an important role in the integration of these two major signalling pathways. In support of this, investigations concerning the role and activation of the JNK have revealed that they are implicated in the responses to growth factors and other environmental stimuli (Coso et al., 1995) and appear to be activated under conditions of cell stress hence their original nomenclature of stress activated protein kinases (SAPK). As well as TCR and CD28 several other surface stimuli have been identified which are able to activate JNK including TNFα, IL-1, UV light and heat shock. Although little is known about the signalling pathway mediated by these molecules it appears to involve the ras- like GTPases Cdc42 and

Rac-1(Coso et al., 1995; Minden et al., 1995). The target of Cdc42 and Rac-1 appears to be a new kinase PAK which in turn activates MEKK, SEK(JNKK) and finally SAPK / JNK. Activation of JNK leads to phosphorylation of the amino terminus of the transcription factor c-jun which is discussed in more detail later.

1.3 CD28 SIGNALLING PATHWAYS

Currently very little is known concerning the signalling pathways activated following CD28 engagement although early studies showed an insensitivity to cyclosporin A (CsA) indicating a calcium independent pathway (reviewed in June et al., 1990). Since like the TCR, CD28 has no intrinsic kinase activity, co-immunoprecipitation of several tyrosine phosphorylated proteins after B7 ligation implied association with a protein tyrosine kinase (PTK). The identity of this kinase is still unknown although the src family kinases are the main proposed candidates (reviewed Rudd et al., 1994; Raab et al., 1995; Lu et al., 1992). Recently, however, several signalling molecules associated with CD28 have been identified and are summarised in figure 1.4.

1.3.1 PHOSPHATIDYLINOSITOL-3-KINASE (PI3 KINASE)

The first important discovery was the detection of PI3 Kinase products following CD28 receptor stimulation (Ward et al., 1993). This led to further investigations and the subsequent demonstration of the association between CD28 and PI3 Kinase (Prasad et al., 1994), hence the proposal of PI3 Kinase as a major participant in the signal transduction pathway of CD28.

PI3 Kinase is a heterodimeric lipid kinase molecule which phosphorylates the membrane lipid phosphatidylinositol on the 3 position of the inositol ring (see figure 1.5). This leads to the generation of a number of different D-3-phosphoinositides including phosphatidylinositol-(3)-monophosphate (PtIns(3)P), phosphatidylinositol-(3,4)-bisphosphate (PtIns(3,4)P_2) and phosphatidylinositol-(3,4,5)-trisphosphate (PtIns(3,4,5)P_3). Whilst all of these molecules have been implicated as messengers in a number of signalling pathways the (3,4,5) trisphosphate form appears to be the most active molecule although the details of its exact targets are still unclear.

PI3 Kinase itself comprises two subunits; the p85 regulatory subunit containing two SH2 domains and one SH3 domain and the p110 catalytic subunit (Hiles et al., 1992). Analysis of target specificity indicate the SH2 domains bind a core phosphotyrosine motif, YMNM such as is found in the cytoplasmic tail of the CD28 molecule (Prasad et al., 1994) and PI3 kinase recruitment to CD28 has been demonstrated in immunoprecipitates of the CD28 molecule following antibody or B7 ligation. The

association is rapid, occurring within 30 seconds of CD28 engagement and has a duration of at least 30 min (Lu et al., 1995). Much of the data concerning CD28 / PI3 Kinase association and function was elucidated with the aid of the enzyme inhibitor wortmannin. This compound, a fungal metabolite, was found to inhibit the catalytic activity of PI3 Kinase by blocking the ATP binding site on the p110 subunit but it does not, however, prevent CD28 recruitment of the kinase (Ward et al., 1995). For a long time the intermediate steps between PI3 Kinase binding and the downstream targets of CD28 signalling remained unknown although with the recent discovery of protein kinase B (PKB) (Burgering and Coffer, 1995) and S6 kinase (reviewed Downward, 1994) the identity of at least some of these targets at last appear to be emerging. Furthermore Rac1 has recently been shown to be another downstream target of PI3 Kinase signalling (Hawkins et al., 1995) and may explain the ability of CD28 to regulate JNK activation.

The serine/ threonine kinase PKB (c-akt), as expected, has sequence homology to the other protein kinases PKA and PKC. It was discovered as the cellular homologue of the viral transforming protein v-Akt and was found to be activated following PDGF and EGF receptor stimulation. Interestingly PKB activation was also found to be sensitive to inhibition by wortmannin, placing it as a possible downstream target of PI3 Kinase. The cell cycle regulator p70S6 kinase is activated by serine / threonine phosphorylation and has been shown to be regulated by both the EGFR and by phorbol esters (reviewed Erikson, 1991). These data taken in combination with the sensitivity of S6 kinase to wortmannin led to the hypothesis that PI3 Kinase activation of PKB via the production of D -3- phosphoinositides may in turn activate p70S6 kinase. Much of this hypothesis is based on studies conducted using constitutively activated and dominant negative mutant PKB constructs (Burgering and Coffer, 1995). Furthermore since it is already known that p70S6 kinase activation can be inhibited by rapamycin binding to its target protein and that phorbol ester stimulation of S6 kinase is rapamycin but not wortmannin sensitive (Chung et al., 1994) the current hypothesis is that PI3 Kinase may activate PKB which in turn triggers target of rapamycin to activate S6 kinase. Further research will be necessary to confirm this sequence and many of the specific details of interactions have yet to be elucidated.

One recent study carried out by Hutchcroft et al (1995) found that treatment of Jurkat cells with PMA actually inhibited the recruitment of PI3 Kinase to CD28 without affecting the downstream effects of CD28 on IL-2 production. PMA was thought to exert its effects by serine/ threonine phosphorylation of a target site on the CD28 cytoplasmic tail close to the PI3 Kinase binding site as demonstrated *in vitro* and was unaffected by the addition of wortmannin. Unfortunately the study was not carried out in normal T cells so it is not possible to establish whether this is a peculiarity of the

transformed cell line or whether it is a feature of all activated cells that PI3 Kinase recruitment is not required for IL-2 production. Previous studies have shown a significant inhibition of proliferation and IL-2 production in resting T cells by wortmannin although activated cells appeared less affected (Ueda et al., 1995). Since IL-2 production was not inhibited in the Jurkat cells this may indicate another role for PI3 Kinase in cellular activation other than in the regulation of IL-2 gene transcription.

1.3.2 THE SPHINGOMYELINASE PATHWAY

A very recent discovery concerning CD28-mediated activation of the enzyme sphingomyelinase, has revealed a possible second signal transduction pathway. Since the sphingomyelin signalling pathway was first described by Okazaki et al in 1989 it has been linked to the signal transducing abilities of several important surface receptors including Fas, TNFα and IL-1β (Cifone et al., 1993; Dressler et al., 1992; Mathias et al., 1993). This appears rather contradictory since, in some circumstances, activation of these molecules has been shown to result in growth arrest and apoptosis whilst CD28 is a potent pro-proliferative, costimulatory molecule. However, since in the presence of additional signals TNF and IL-1 have also been shown to be stimulatory (Smith et al., 1994; Muegge et al., 1989) it appears that the outcome following activation of this pathway may be more complex than at first thought and numerous studies are now being carried out to try and resolve this issue.

The sphingomyelin pathway, summarised in figure 1.6, originates from the membrane sphingosine- based phospholipid, N- acylsphingosin- 1- phosphocholine which undergoes hydrolysis by a phospholipase enzyme, sphingomyelinase. It is this enzyme which is activated following stimulation of the surface signal transducing molecules although the actual mechanism of interaction between the receptor and the enzyme has yet to be elucidated. So far two different forms of the sphingomyelinase enzyme have been described (Kolesnick, 1991). A membrane- bound neutral form which is dependent upon magnesium was the first to be discovered and is activated by IL-1. A second form which is magnesium independent is found sequestered in the acidic lysosomes. However, since the hydrolysis reaction is proposed to take place at the membrane the maintenance of the acidic environment for the latter enzyme does not seem possible and has raised speculations of receptor internalisation and endosomal acidification (Kolesnick and Fuks, 1995). A recent study by Boucher et al (1995) reported that the acidic form of the enzyme was activated by CD28. Interestingly TNF has recently been shown to activate both forms of the enzyme (Wiegmann et al., 1994) although a correlation between the enzymatic form activated and the various effects of TNF has not yet been elucidated.

1.3.3 THE DOWNSTREAM TARGETS OF CERAMIDE

The outcome of sphingomyelinase activation is the generation of phosphorylcholine and ceramide; the latter being the active signalling entity. The number of targets for ceramide appears to be increasing rapidly as the unravelling of the pathway continues but it is generally agreed that it can activate at least four downstream molecules. The first is the guanine nucleotide exchange protein, Vav which has sequence homology to other members of the family such as the ras- activating protein, SOS (Gulbins et al., 1994). In addition to its nucleotide exchange capabilities Vav also bears a lipid binding domain similar to that found on PKC for DAG and phorbol esters. The significance of this has yet to be understood but it raises the possibility of a link between the CD28-mediated sphingomyelin pathway and the TCR mediated MAPK cascade. A second target for ceramide is a 97kDa proline directed serine/threonine protein kinase named CAPK (Kolesnick and Golde, 1994; Mathias et al., 1991). This is a membrane bound kinase which has been shown to bind TNF and IL-1 receptors with high affinity. One identified target for this kinase is the proto-oncogene Raf1 (Yao et al., 1995); forming yet another link with the MAPK cascade. The third ceramide target, in contrast to the last, is a phosphatase of the serine /threonine class 2A family, CAPP (Dobrowsky and Hannun, 1992). The active molecule comprises a heterotrimeric complex of which the β subunit is essential for phosphatase activity. CAPP is proposed as the mediator of the apoptotic signals of ceramide since its activation closely matches the ceramide induced apoptotic events and it can be inhibited by okadaic acid (Hannun, 1994). The final target for ceramide is the atypical PKCζ (Lozano et al., 1994). As mentioned previously this PKC isotype is unresponsive to DAG and phorbol esters but appears to mediate the phosphorylation and hence subsequent degradation of IκB, the inhibitory subunit of the NFκB transcription factor complex. Degradation of IκB allows free translocation of the NFκB to the nucleus where it participates in regulation of IL-2 gene expression.

With so many different targets delivering both proliferative and apoptotic signals it is difficult to understand how the ceramide pathway can be rationalised. However, recent discoveries of the inhibitory ability of DAG on ceramide-mediated apoptosis has suggested one possibility Whilst pro-apoptotic agents such as Fas and TNF activate ceramide only, TCR signals in conjunction with a costimulatory signal as would be encountered during an immune response, would activate both DAG and ceramide. Furthermore several studies have shown a protective effect of PKC activation in the prevention of apoptosis induced by a variety of stimuli. This indicates that DAG may have a protective role by preventing the transduction of the ceramide-mediated apoptotic signals whilst allowing transduction of the proliferative signals. Therefore the outcome of T cell stimulation may rely on the relative balance between the protective TCR-induced DAG and the costimulaton-mediated induction of ceramide.

As originally observed there are several points on the CD28 signalling pathway which allow overlap and synergy with the TCR pathway. However, the recruitment of the sphingomyelinase pathway and the activation of PKCζ does appear at present to distinguish the two and may be the one specific marker of the CD28 pathway. Thus a synergy of the two signalling pathways would be required to induce full activation of the cell. This is supported by the observation that stimulation of the TCR alone often induces anergy whilst activation of the sphingomyelinase pathway frequently causes apoptosis. Simultaneous delivery of both signals, however, satisfies all the requirements of activation and proliferation.

1.4 TRANSCRIPTION FACTORS

At whichever point the TCR and costimulatory pathways intersect, one outcome of receptor stimulation is the induction of interleukin-2 gene transcription, resulting in the production and release of the main T cell proliferative cytokine IL-2. The means by which this is achieved is via the complex series of signalling steps discussed above and which culminate in the regulation of DNA binding proteins. These regulatory proteins or transcription factors, bind to the promoter regions of genes 5' to the start site and regulate the transcription of the gene. Their actions are usually to promote gene transcription although some inhibitory transcription factors have been identified (Nakabeppu and Nathans, 1991). Rather than being single proteins transcription factors are often complexes with modular arrangements resulting in the formation of distinct domains. The DNA binding domain often consists of an a helix motif surrounded by or incorporating several positively charged amino acid residues. A second domain, the transactivation domain, mediates cooperative interactions between transcription factor complexes. Transcription factors are not generally functional as isolated units but instead interact with other adjacent factors with the result that the entire promoter region when occupied by a full set of transcription factors, acts as a coordinated regulator of the gene.

In the next few paragraphs the structure, activation mechanisms and regulation of transcription factors in general will be discussed followed by more specific features concerning the transcription factors involved in the regulation of the interleukin-2 gene. The single distinguishing feature of a transcription factor which separates it from other signalling and enzymatic proteins is its ability to bind specific sequences of double stranded DNA. These sequences, ranging from 5 to 20 amino acids, have largely been conserved through evolution and several transcription factor complexes such as NFκB appear to be ubiquitous whilst others such as NFAT appear to be lineage specific.

1.4.1 STRUCTURE

Most transcription factors appear to fall into one of four structural categories depending upon the mechanism of their DNA binding domain, these being helix-turn-helix (HTH), zinc finger, leucine zipper and helix-loop-helix (HLH) (Papavassiliou, 1995). A structural diagram of the four motifs is shown in figure 1.7. The first motif is the HTH which is found in a diverse range of transcription factors, some of which are essential for the differentiation of haemopoietic precursor cells. The HTH motif is found within the homeodomain and consists of three consecutive α helices. Adjacent domains can interact with and influence the DNA binding ability and functional activation of the complex. A relevant example of an HTH motif transcription factor is found in the POU family comprising the Octamer proteins discussed below.

The second motif which functions as a dimer, is the zinc finger motif, found in a wide range of transcription factors including the oncogene bcl-6, the tumour suppressing protein WT1 and the erythroid differentiation factor GATA-1. This motif consists of either a pair of histidines and a pair of cysteines or two pairs of cysteines coordinating a zinc ion with the DNA binding being mediated by specific residues at the base and body of each loop.

The third category has the unusual name of the leucine zipper motif based on the observation of two transcription factors zipping together by interlocking of the leucine ' teeth '. An α helix forms the backbone of the DNA binding domain, every seventh amino acid of which is a leucine. The complex formed by the zipping together of the homo or heterodimeric units results in a stable structure exhibiting the correct configuration for DNA binding. An example of such a complex is found in the AP-1 transcription factor comprising the leucine zipper proteins fos and jun.

Finally there is the HLH group comprising members which bind immunoglobulin gene enhancers such as the c-myc factors involved in growth regulation. These show much similarity to the leucine zipper factors with a positively charged region responsible for DNA binding and an adjacent dimerisation domain.

1.4.2 ACTIVATION

An alternative classification system involves the differences in modes of activation of the transcription factors. They can be broadly subdivided into one of three activation mechanisms : a) those factors that are activated by protein kinases which translocate from the cytoplasm to the nucleus following receptor stimulation; b) those factors

which are present in the cytoplasm and undergo phosphorylation to induce their own translocation to the nucleus and c) those factors which are released from an anchor or inhibitory binding protein and are then able to translocate. However, these categories are not mutually exclusive as some transcription factors can be activated by multiple pathways. One example is c-fos, a transcription factor which functions in a complex with c-jun proteins to form the AP-1 transcription factor. The c-fos gene itself is under regulatory control by several cis- acting DNA elements including a serum-response element (SRE), the c-SIS- inducible element (SIE), an AP-1 binding site and a cAMP response element (CRE) (reviewed in (Edwards, 1994)) as shown in figure 1.8. The SRE alone contains binding sites for several different transcription factors including those for a serum response factor (SRF), a site for Elk-1, a member of the Ets family of proto-oncogenes and nuclear factor - IL-6 which appears to link the SRE to the AP-1 binding site. Binding of Elk-1 appears to be dependent on the presence of bound SRF and data indicate it is this ternary complex (TCF) which is regulated by signals from the MAP kinase pathway (Whitmarsh et al., 1995). This provides an example of the first method of transcription factor activation. Following receptor stimulation, activation of the MAP kinase signalling pathway results in the rapid (5-10 min) translocation of active ERK and p90rsk S6 kinase to the nucleus where they phosphorylate and hence activate the Elk-1 and SRF factors respectively.

The additional signalling events required for the transcription of c-fos provide an example of the second mechanism of transcription factor activation and involve the activation of a family of latent cytoplasmic factors known as signal transducers and activators of transcription (STAT) proteins (Edwards, 1994; Brunn et al., 1995). Discovery of these proteins is relatively recent and the nomenclature of the different family members has not yet been formalised. However, all are activated via tyrosine phosphorylation probably involving a member of the Janus kinase family (JAK1, 2, 3 or TYK2) (Asao et al., 1994; Bacon et al., 1995; Wilks and Harpur, 1994). These lie downstream of a number of IFN, growth factor and cytokine receptors including IFN α and β, IL-2, IL-4 and IL-12. Since this pathway is independent of ras activation it can be considered as a completely separate activation signal from that provided via MAP kinase. Phosphorylation of STAT proteins is thought to result in a conformational change allowing their dissociation from the surface receptor, dimerisation and their translocation to the nucleus where they bind specific DNA sequences in gene promoters. In the case of c-fos STAT proteins have been shown to associate with another factor named p48 and together they bind the SIE upstream of the SRE and participate in the regulation of c-fos transcription (Edwards, 1994).

The third mechanism of activating transcription factors is typified by the NFκB transcription factor complex which, in unstimulated cells, remains sequestered in the

cytoplasm bound to an inhibitory protein IκB. Activation of the cell results in the phosphorylation and subsequent degradation of this inhibitory protein so releasing the NFκB which translocates to the nucleus to effect its actions. Since NFκB has a major role to play in IL-2 gene regulation the remainder of the activation of this type of transcription factor will be discussed below.

1.5 THE IL-2 GENE PROMOTER

The IL-2 promoter extends approximately 300bp upstream of the 5' start site from -326 to +46bp (Jankevics et al., 1994). Within this region are encoded the binding sites for between six and eight identifiable transcription factor binding sequences (see figure 1.9). The number of factors varies depending upon the cell types used for the experiments and whether complex sites are considered as single or separate units. Generally single binding sites have been identified for NFAT and NFκB, whilst two AP-1 and two Oct sites have been demonstrated. A seventh site labelled the CD28 response element (CD28RE) shows much similarity to the NFκB site discussed below. The existence of multiple sites does not, however, mean that all are functional and in fact DNAse I footprinting of the IL-2 promoter only detected protein protection at the proximal and not the distal AP-1 binding site (Durand et al., 1988). Whether the other sites arose simply by DNA duplication or whether they have a relevant function is still unknown.

The generation of IL-2 requires the occupancy of all the functional transcription factor binding sites. Fiering et al (1990) discovered that a threshold concentration of the NFAT transcription factor was required in the promoter region before transcription was activated. Although many transcription factors appear to be driven by PKC activation and calcium alone (ie TCR signals) costimulatory signals may provide the enhancement required to synthesise sufficient transcription factor to overcome the threshold barrier. The purpose of threshold levels is currently unclear but may either regulate the specificity of which gene is activated by having different transcription factor thresholds for different genes or the purpose may be to ensure that IL-2 transcription only occurs in response to a strong antigen-induced challenge.

1.5.1 AP-1

AP-1 is a ubiquitous transcription factor which is involved in the regulation of many genes concerned with cell growth and differentiation. Binding of the complex to the IL-2 promoter has been demonstrated in both mature $CD4^+/CD8^-$ and immature $CD4^-$

/CD8$^-$ thymocytes but not during the intermediate CD4$^+$/CD8$^+$ stages when the cells are undergoing selection. The AP-1 complex binds to a highly conserved DNA sequence termed the TPA-responsive element (TRE) (TGAGTCA) due to its binding capabilities of phorbol ester-induced complexes. The activation requirements for AP-1 have been somewhat confused by the use of transformed cell lines such as Jurkats which often show some constitutive activity. Furthermore Los et al (1994) reported that AP-1 activity could be induced in Jurkat cells by stimulation with PMA alone and that this activity was downregulated by the addition of antiCD28 antibodies. In contrast, an alternative report by Rincon and Flavell (1994) on the induction of functional AP-1 activity in primary mouse T cells demonstrated a requirement for full TCR activation and costimulation. In general it appears that production of a transcriptionally active AP-1 complex occurs in two or more stages. PMA stimulation of the cell can induce DNA binding of the AP-1 complex but this has no functional activity. Calcium and costimulatory signals are then required for post translational modification of the complex to make it functionally active. Serine/ threonine phosphorylation sites have been identified at both the amino and carboxy terminals of the c-jun subunit of the AP-1 complex and the amino terminal site appears to be under the regulatory control of the MAP-family kinase JNK (Hibi et al., 1993). Phosphorylation of serine 63 and 73 of c-jun leads to an increase in the transcriptional activity of the complex. Interestingly JNK has been proposed as one of the downstream targets of CD28 activation and it therefore may explain the requirement for costimulatory signals in the post translational modification. Phosphorylation of the carboxy terminal site of c-jun appears to have an inhibitory effect on DNA binding. This phosphorylation on serine 243 and 249 and threonine 231 appears to be mediated by casein kinase II or GSK3 but is reversed after phorbol ester treatment which induces DNA binding. The mechanisms controlling the phosphorylation and dephosphorylation of these sites is still unknown as the kinases themselves do not appear to be phorbol ester regulated and the existence of a candidate phosphatase has not yet been proved. There is some speculation that there is no enzymatic regulation but a conformational regulation instead and that phosphorylation of the amino terminal sites causes a conformational change in the c-jun subunit reducing the accessibility of the carboxy terminal sites to the phosphorylating kinases (Papavassiliou and Bohmann, 1995).

The AP-1 complex is a dimer comprising members of the c-jun and c-fos protein families. Though c-jun homodimers will bind and activate TREs the heterodimeric complex of fos and jun has a considerably higher binding affinity. Several members of each family have been identified and multiple dimeric complexes have been shown to be functional. The c-fos family comprises the members c-Fos, FosB, Fra-1, Fra-2 and FosB2 whilst the jun family consists of c-Jun, JunB and JunD, however, the elements FosB2 and JunB have been shown to exhibit some inhibitory effects (Nakabeppu and

Nathans, 1991; Chui et al., 1989). The subunits associate via a leucine zipper motif which brings the basic amino acids together to allow DNA binding. This is mediated via cysteine residues which are required to be in the reduced state (i.e. -SH) (Abate et al., 1990). This may explain why AP-1 binding and activation is induced in antioxidant conditions and inhibited in the presence of oxidative compounds. Furthermore, Handel et al (1995) proposed that DNA binding of AP-1 could be inhibited by the presence of gold thiol compounds due to an interaction between the gold and the cysteine residues on fos and jun. This may underlie the rationale behind the use of gold compounds in the treatment of rheumatoid arthritis due to the inhibition of AP-1 induction of inflammatory cytokines.

1.5.2 OCT

A second ubiquitous transcription factor associated with IL-2 gene transcription is Oct-1, a constitutive member of the POU domain multigene family, which recognises and binds several degenerate sequences based around the motif ATTTGCAT (Bamruker et al., 1988). Two putative binding sites have been identified within the IL-2 promoter; one at position -63 to -93 and a second at -242 to -256. Although present in resting cells the Oct-1 protein requires activation by signals from the TCR before becoming functional and does not respond to PMA alone (Durand et al., 1988). Further studies of the binding requirements for Oct-1 at the proximal site revealed that it actually functions as a complex with an octamer associated protein (OAP). This has since been identified as AP-1, which explains the requirement for TCR stimulation and the sensitivity of the complex to CsA (Ullman et al., 1993). This complex is separate from the AP-1 binding site further upstream but it appears both are required for IL-2 transcription since mutation in either site abrogated IL-2 production. A similar site was observed in the promoter region of the IL-4 gene but in this case the binding sites for Oct-1 and AP-1 were separated by a binding site for NFAT (Pfeuffer et al., 1994). Analysis of the activation requirements for the IL-4 promoter, however, revealed that binding of NFAT actually inhibited Oct binding indicating the latter may function as a negative regulator of this cytokine.

A second inducible factor of the same family has been identified and named Oct-2 (Kang et al., 1992). This factor was detected in B cells and peripheral blood T cells but only in some T cell lines and not in Jurkats (Staudt et al., 1988). The induction of Oct-2 is first observed about 3 hours after stimulation of the cell indicating it may play a role in the long term changes in gene expression rather than cytokine regulation.

1.5.3 NUCLEAR FACTOR of ACTIVATED T CELLS (NFAT)

The existence of the NFAT transcription factor was known for a long time before the identity of its components were discovered and it was originally thought to be the T cell specific factor which differentiated T cell cytokine production from other cells bearing gene regulating promoter sequences. However, it has since been shown that NFAT is not unique to T cells and is also expressed in B cells and several other immune sites (review Hoey et al., 1995). Considering the ubiquitous nature of the majority of the transcription factors the question arises as to how one gene is specifically activated without switching on others regulated by the same factors. One answer may lie in the subtle differences in protein isotype within the complexes or as discussed previously in the threshold concentrations of the factors. Furthermore whilst NFAT induction in T cells has been demonstrated at two hours it could not be detected in B cells until 24 hours after stimulation (Verweij et al., 1990). Thus NFAT may have completely different roles in different cell types.

NFAT comprises two components; an inducible nuclear component which appears to be PMA regulated and a constitutive but inactive cytoplasmic component which requires the presence of calcium for its activation and subsequent translocation to the nucleus (Northrop et al., 1993). Here the two subunits combine to form a single complex which associates with a DNA binding site positioned at -255 to -285 in the IL-2 promoter. Similar binding sites have been observed in the promoter regions of several other T cell cytokine genes including IL-4, IL-5, IFNγ and GM-CSF (review Rao, 1994). The nuclear subunit has now been identified as AP-1 which explains its PMA inducibility. Much work has been undertaken to identify the fos and jun members involved and whilst one group identified Fra-1 and JunB as the major factors (Boise et al., 1993a) it now appears that like AP-1 itself several different heterodimeric complexes can carry out this role. In the IL-2 gene promoter the complex binds as a single entity whilst in the IL-4 gene the two units appear to bind adjacently in a cooperative manner but can be considered as separate (Rooney et al., 1995). However, this work was carried out in murine cells and may not extend to the human gene promoter.

The cytoplasmic unit, referred to here as NFAT, is now known to comprise a family of proteins each containing a DNA binding site and a specified region bearing limited homology to the Rel domain of κB proteins (Nolan, 1994). Three members of the family were originally identified and labelled NFATc, p and x (Northrop et al., 1994; Masuda et al., 1995). More recently another group has identified a further two members NFAT3 and NFAT4 (Hoey et al., 1995). Although shown to be different from NFATc

and p it remains to be seen whether either correspond to NFATx. The reason for the multiplicity of cytoplasmic units is currently unclear as only NFAT3 has been demonstrated to have a significantly different binding requirement and tissue distribution but further research may resolve this issue. At present all forms can effectively be considered as having identical properties in that their activation requires a calcium signal initiated by TCR engagement. Calcium binds cooperatively to a binding

protein, calmodulin and it is this complex which activates the phosphatase calcineurin which is responsible for the dephosphorylation of the cytoplasmic NFAT. Once dephosphorylated the NFAT subunit can translocate to the nucleus and participate in gene transcription. This sequence of events is a major activation pathway and it is the target of the immunosuppressive agents CsA and FK506 (McCaffrey et al., 1993). Association of these agents with their respective binding proteins, cyclophilin and FKBP, enables their association with and inhibition of calcineurin resulting in a very effective inhibition of IL-2 generation and hence the immune response thus indicating the vital role played by NFAT in the activation process.

1.5.4 NFκB

The NFκB transcription factor was first discovered associated with the promoter region of the immunoglobulin light chain, kappa and was thought to be B cell specific (Sen and Baltimore, 1986). It has since been shown to be a ubiquitous factor, occurring in the promoter regions of a number of inflammatory cytokines. It is also utilised by several viral pathogens to subvert the cells machinery into the production of viral proteins. One such virus is the human immunodeficiency virus which contains two NFκB elements in the long terminal repeat region (Nabel and Baltimore, 1987).

NFκB is a heterodimeric complex consisting of two members of the Rel family of transcription factor proteins. Five members have been identified so far; c-Rel, RelA (p65), RelB, NFκB1 (p50) and NFκB2 (p52). All contain a N terminal Rel homology domain of approximately 300 amino acids and can interact to form a variety of homo and heterodimeric complexes including the p65/ p50 heterodimer, which is usually considered as the standard NFκB complex. The NFκB binding sites are based on the sequence GGGACTTTCC but a considerable amount of degeneracy has been observed in different gene promoters and it may be that different dimeric complexes show binding preferences to different sequences introducing some specificity to gene activation. The structure of the p50 homodimer has been studied and has revealed the N terminal Rel homology domain consists of two β-barrel domains which grip the DNA in the major groove (Muller et al., 1995a). It is proposed that when solved, the structures of the other functional dimers will reveal similar binding mechanisms.

NFκB binding can be demonstrated in Jurkat cells following PMA stimulation alone although like AP-1 this does not result in a functional complex. A second signal appears to be required indicating a role for modification of at least one of the components. NFκB occurs as an inactive cytoplasmic complex bound to an inhibitory anchor protein IκBα (Baeuerle, 1991). This is one of a family of proteins characterised by their ability to bind and inhibit the effects of the Rel proteins and which contain a

series of ankyrin domains conferring a specific structural motif. IκBα associates with the NFκB complex via the C terminal domains of the transcription factor in such a way as to mask the nuclear localisation signals of the NFκB complex (Beg et al., 1992). This prevents its translocation to the nucleus and hence its effect on gene transcription. It has been shown that nuclear NFκB activity can be induced following stimulation of the cells with a number of agents including PMA, TNF and IL-1(Baeuerle, 1991). This is achieved by phosphorylation and degradation of the IκBα protein allowing free translocation of the NFκB. Although the actual mechanism by which this occurs is not fully understood recent studies by several groups have supplied some of the details. Chen et al (1995) revealed that the ubiquitin- proteasome pathway may be involved in the removal of IκBα whilst Brockman et al (1995) showed that the degradation depended upon the presence and phosphorylation of two serine residues in the N terminal domain of the IκBα molecule. Interestingly it was found that neither phosphorylation nor ubiquitination were sufficient to induce dissociation of the IκBα from the NFκB complex; an event which required the binding of the 26S proteasome. IκBα levels were shown to fall 2 to 5 minutes after PMA treatment and remained depressed for an hour (Henkel et al., 1993). Reappearance of cytoplasmic IκBα follows gene transcription and protein synthesis which interestingly is initiated via NFκB (Brown et al., 1993) therefore indicating that NFκB can regulate its own activity via an autoregulatory feedback mechanism. Agents generating oxidant conditions have also been shown to activate NFκB (Los et al., 1994 and 1995) and this is thought to be mediated via reactive oxygen intermediates (ROIs) which may also regulate IκBα degradation although the mechanism is unclear. This is opposite to the generation of the AP-1 transcription factor which is enhanced by antioxidants and appears rather contradictory since both are required for IL-2 gene transcription. However, the redox regulation may be more important in the expression of other genes which are only regulated by one or other of the two factors. Both AP-1 and NFκB are, however, regulated by glucocorticoids. These are reagents which have been used routinely for several years in the treatment of chronic inflammatory diseases and yet their mode of action was not fully understood. It was known that glucocorticoid drugs could bind steroid receptors in the cytoplasm, translocate to the nucleus and bind as transcription factors to DNA binding sites (review Marx, 1995). However, many of the genes repressed by glucocorticoid treatment did not have binding sites in their promoter

regions. It was noted, however, that the drugs also down regulated AP-1 activity but the extent of this was insufficient to account for the full effect of the drugs. Recently two groups have shown that glucocorticoids can also inhibit NFκB activity (Auphan et al., 1995; Scheinman et al., 1995). This is mediated via an increase in IκBα transcription which results in a large increase in the cytoplasmic levels of the inhibitory protein. Thus as soon as the NFκB is released from one IκBα molecule it is bound by another before it can reach the nucleus. Since NFκB is involved in the transcriptional regulation

of a number of inflammatory cytokines this could explain why the glucocorticoid drugs are so effective as immunosuppressants. It may also help in the design of more specific drugs which will inhibit inflammation without the many other side effects of the glucocorticoids.

1.5.5 CD28RC

The CD28 response complex (CD28RC) is a new transcription factor which binds to the IL-2 promoter at position -154 to -164 and which was first described by Fraser and Weiss in 1991. However following the report that the complex may comprise the Rel family members it has been proposed as an extra NFκB complex rather than a new transcription factor. Ghosh et al (1993) investigated the complex and reported the presence of c-Rel, Rel A and NFκB1 proteins which together bind a sequence on the IL-2 promoter bearing some homology to the NFκB binding sites. However, this report is currently disputed by a second group who could find no evidence for the binding of NFκB family proteins to this site (Verweij, personal communication). The CD28RC was originally described as a CD28-induced factor but although stimulation of CD28 enhances its expression, ligation of the surface receptor is not essential for transcription factor induction and one group has induced the complex using PMA alone (Bryan et al., 1994). Unfortunately much of the information concerning the induction and function of this complex has been confused by the use of transformed cell lines which can be activated independently of costimulatory signals, however, the few studies carried out in peripheral blood T cells did give some insight into the signalling pathways involved in the generation of this complex. It is difficult to distinguish CD28RC induction from the induction and regulation of NFκB complexes other than by the indication that the CD28RC involves a ternary complex which appears to assemble in the nucleus rather than being present in an inactive form in the cytoplasm. Stimulation of peripheral blood T cells with PMA and αCD28 antibodies resulted in an increase in the nuclear translocation of c-Rel compared to that observed with PMA treatment alone (Bryan et al., 1994). This was accompanied by a prolonged downregulation of IκB of up to 12 hours compared to the usual 1 hour although the mechanism for this is not yet known.

Furthermore CD28 costimulation induces an increase in the phosphorylation of the c-Rel protein which appears to be required for functional activity of the complex.

A second study by Los et al (1995) showed that ligation of CD28 resulted in an increase in ROIs and an induction of CD28RC and NFκB complexes. The former was measured as an increase in the intracellular levels of hydrogen peroxide with a concomitant decrease in the antioxidant glutathione. The linkage between the CD28 receptor and the induction of ROIs is thought to be mediated via protein tyrosine kinases since preincubation with the PTK inhibitor, herbimycin abrogated the response. One reported downstream target of PTKs is phospholipase A_2 which cleaves arachadonic acid to generate the precursors of prostaglandins and leukatrienes. Whilst inhibitors of the prostaglandin pathway had no effect, a 5- lipoxygenase inhibitor blocked the downstream activation of CD28RC and NFκB by CD28, implying this enzyme plays a role in their generation. This further extends the link between redox reactions and transcription factor regulation although the reason for this and its significance still eludes us.

Interestingly, a second agent which appears to have immunosupressive effects is the protein kinase A activator, forskolin. Although not used clinically, laboratory experiments have indicated that it inhibits the translocation of NFκB complexes by retarding IκB degradation (Neumann et al., 1995). In addition PKA activation produces an increase in the non-NFκB regulated cytokine IL-4 which exerts an anti-inflammatory effect. *In vivo* PKA is known to be activated by prostaglandin E (Phipps et al., 1991), an additional product of the enzyme phospholipase A2. Thus PKA may be activated in parallel and could act as a physiological regulator of CD28 signalling and IL-2 production. Furthermore PKA has been shown to inhibit the interaction between ras and raf on the mitogen activated signalling pathway (Wu et al., 1993) and may thus prove to be an important negative regulator of T cell signalling.

1.6 SUMMARY AND AIMS

It is evident from the summary above that whilst we have a basic understanding of the pathways involved in the transduction of a signal from a surface receptor to gene regulation in the nucleus, much of the detail is still missing. Furthermore although CD28 activation is known to be a critical step in this activation process its specific role is far from clear. Does CD28 activate specific signalling pathways or is it just an enhancer of TCR derived signals ? Certainly much of the current work points to a considerable overlap of different signalling pathways and one of the major problems to

date has been the inability to completely separate the signals emanating from the two receptors and therefore to study the role played by CD28 in isolation.

Previous studies have shown a global requirement for CD28 costimulation in the activation of resting T cells although the identity and specific role of the CD28 derived signals has not been fully addressed. The initial aim of this work was therefore to establish *in vitro* assays to determine the requirement for and function of CD28 signals in specific T cell activation events. These were assessed using proliferation assays to

highlight the role of CD28 in cell cycle progression and cytokine production and transcription factor detection assays as a signal transduction endpoint. Although some reports have indicated a role for CD28 in the modification of the IL-2 regulatory transcription factors the information is rather fragmented and has largely arisen from stimulation of transformed cells in the presence of PMA.

Even more fragmented is the information surrounding the identity of the signal transduction pathways associated with CD28. As to the reported involvement of PI3 Kinase and acidic sphingomyelinase in CD28 signalling, although both enzymes have been proposed to be activated following CD28 ligation, the relative contributions and main role of each pathway in the activation process is still unclear. Further investigation is obviously required to establish whether the pathways are activated simultaneously and if so whether they act independently or synergistically to mediate a response.

The use of mitogenically-activated transformed cell lines as models of T cell activation has provided a considerable amount of data on the receptor-to-nucleus signal transduction pathways and T cell activation events. However, several assays have also indicated that the responses shown by these cells are not always an exact representation of the responses in normal T cells due to the presence of constitutively activated pathways in the transformed cells. Using the knowledge gained from these initial studies it is therefore now necessary to develop similar studies using normal T cells activated by physiological stimuli to provide more relevant results.

The following report describes the work carried out in the pursuit of these aims and involves the study of cellular responses in both resting T cells and in the established T cell model, Jurkat cells. Whilst these two models provided some information on the requirement for costimulation and its overall effects on T cell activation, the ability to study the effects of CD28 signalling was considerably enhanced by the development of a new T cell model. This model, based on previously activated quiescent T cell blasts, was responsive to B7 stimulation alone in the absence of concomitant TCR engagement

and enabled the study of the effects of CD28 ligation both in terms of downstream targets and signalling pathways activated, in relative isolation. Although this model was not a exact representation of a resting T cell it retained the characteristic feature of requiring two signals for full activation and unlike the Jurkat cells responded normally to mitogenic and antibody stimulation. B7 stimulation of these T cell blasts provided some important new information on CD28 mediated signalling and may still prove a useful tool in future studies .

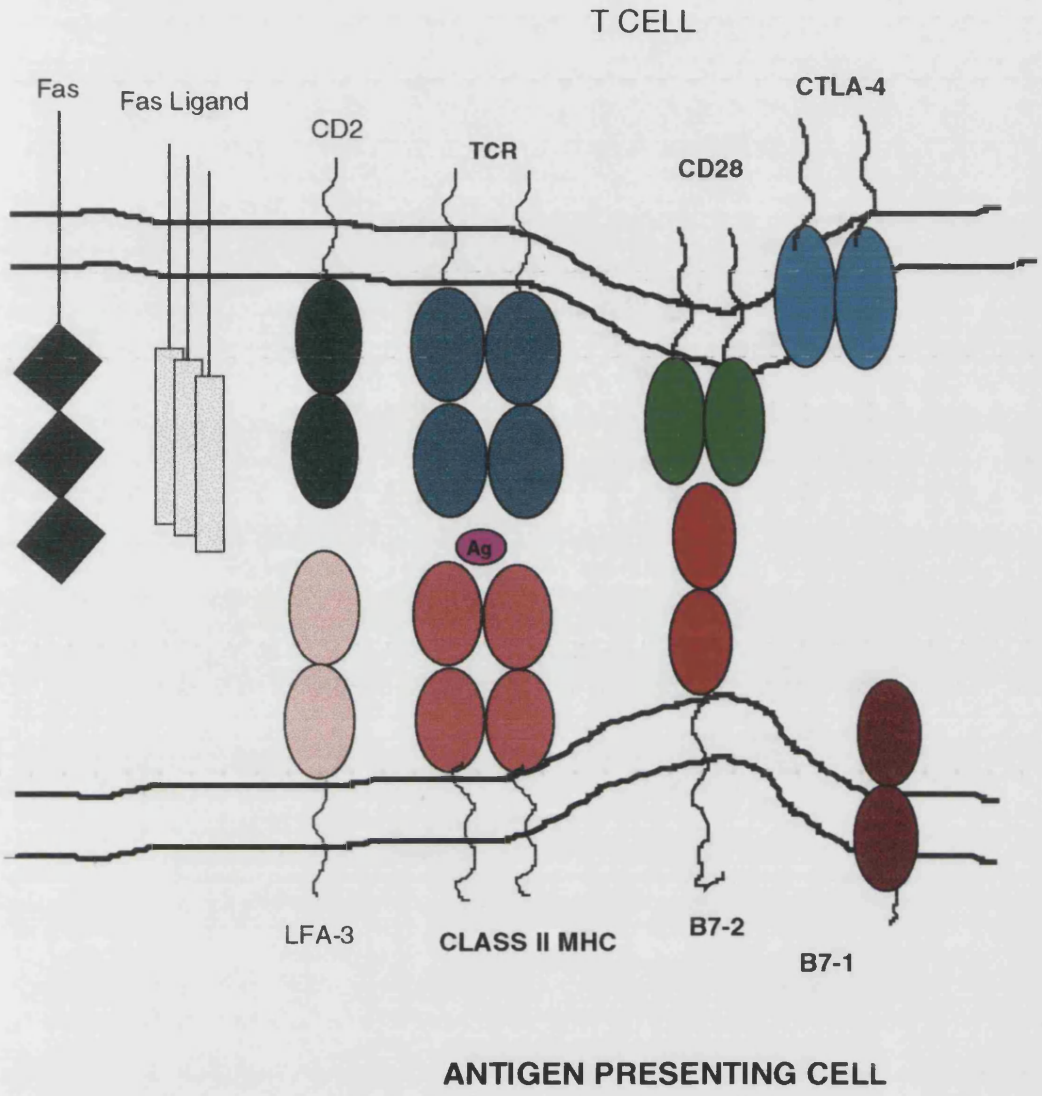


Figure 1.0 A simplified diagram showing the interactions between the main T cell and APC surface molecules involved in T cell activation

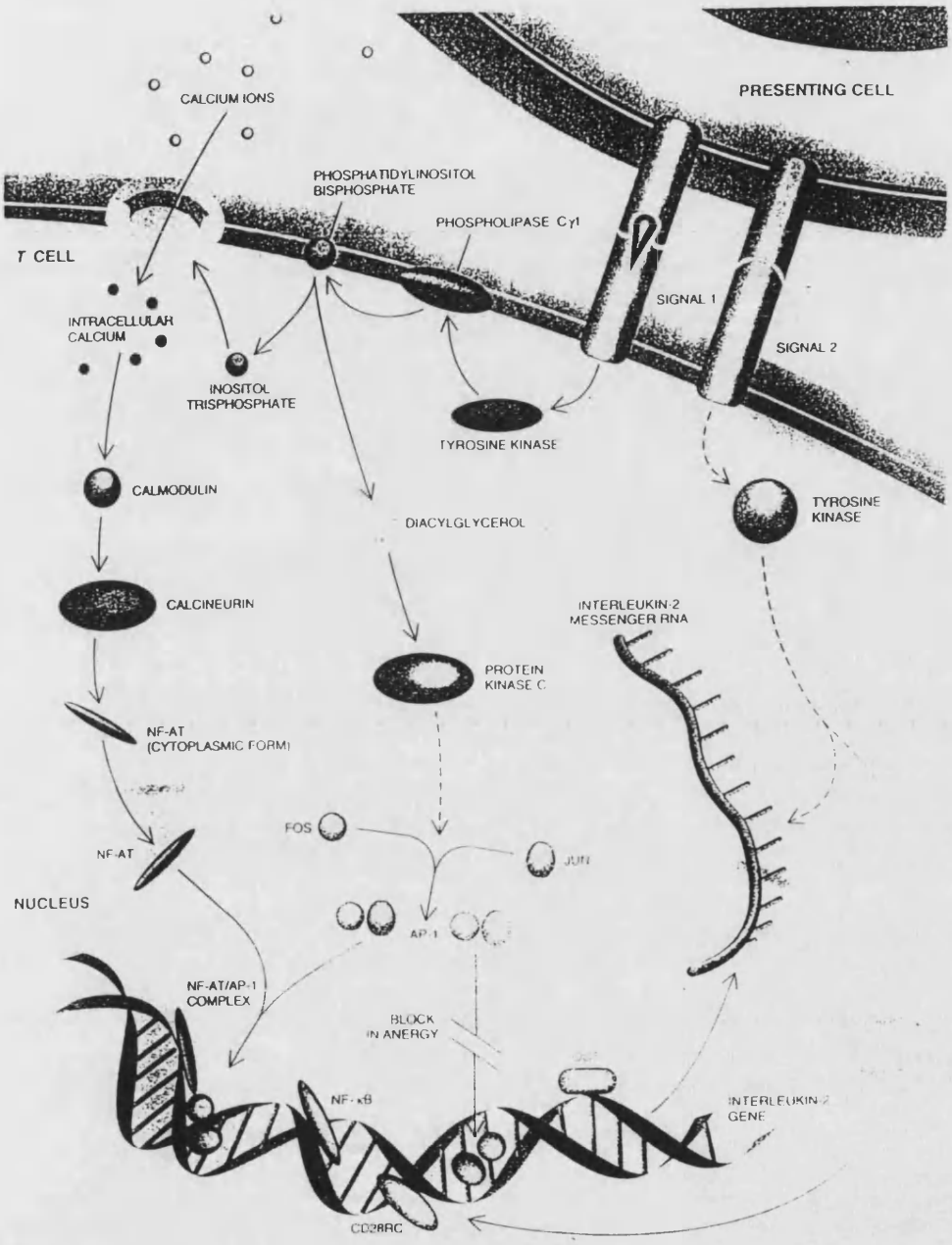


Figure 1.1 A simplified diagram showing the main signal transduction pathways originally thought to be involved in T cell activation (reproduced from ' T Cell Anergy '{Schwartz})

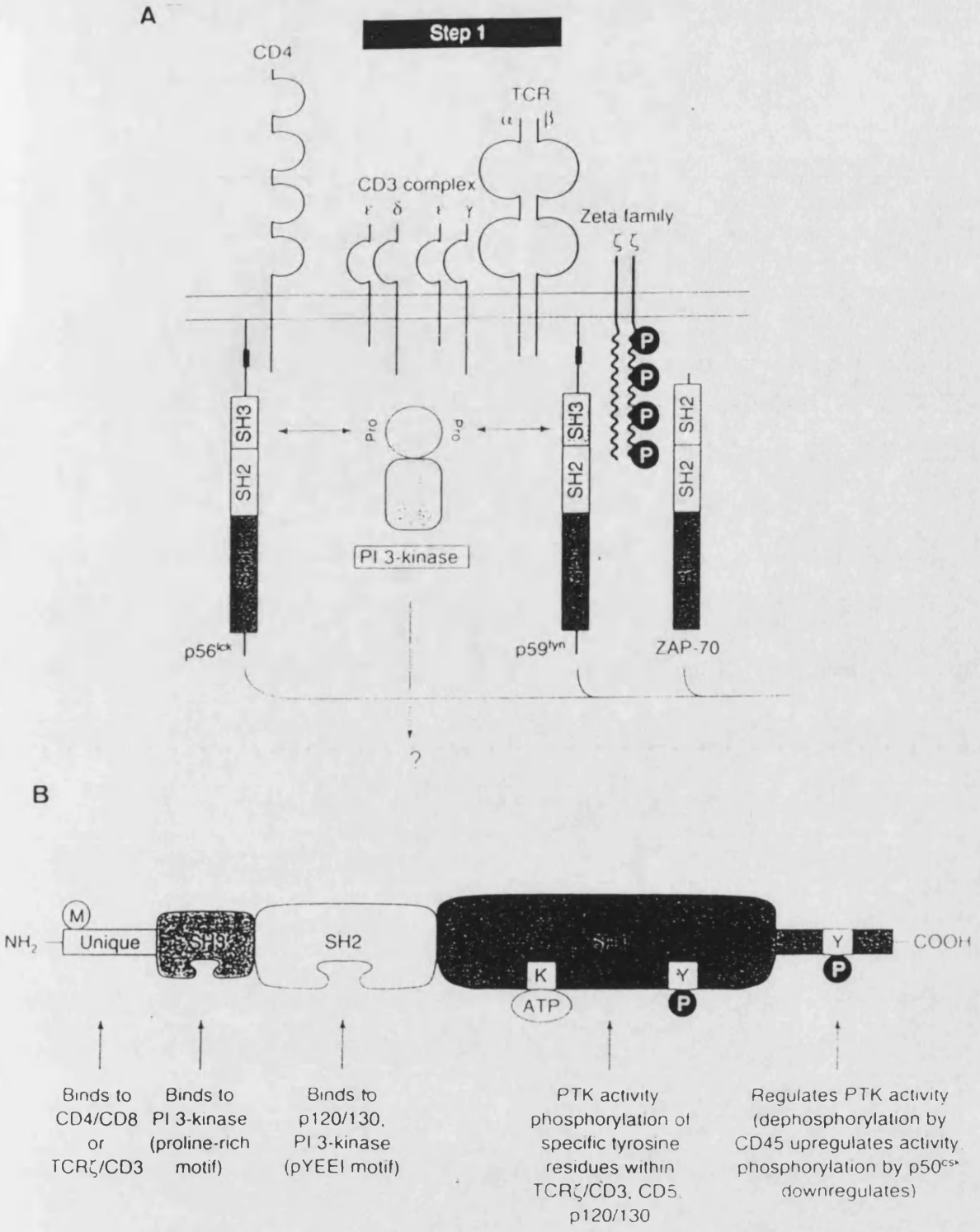


Figure1.2 The association between the TCR/ CD3 complex and protein tyrosine kinases

Figure A shows the relative associations of different PTKs with different members of the TCR/ CD3/ CD4 complex in T cells whilst figure B details the functions of the different domains of the p56lck PTK.(Reproduced from a review by {Rudd})

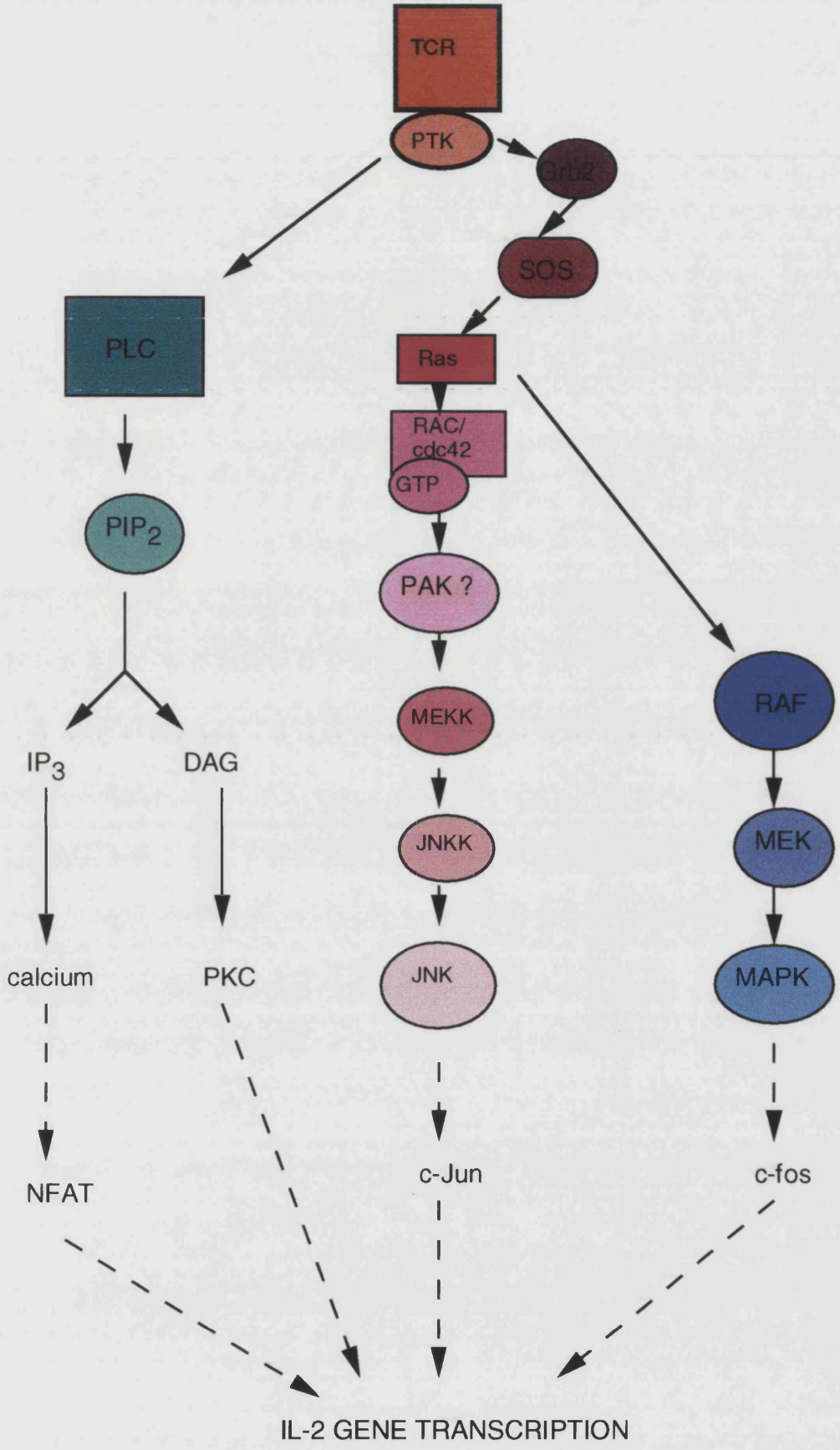


Figure 1.3 A diagramatic representation of the current understanding of TCR-associated signalling pathways

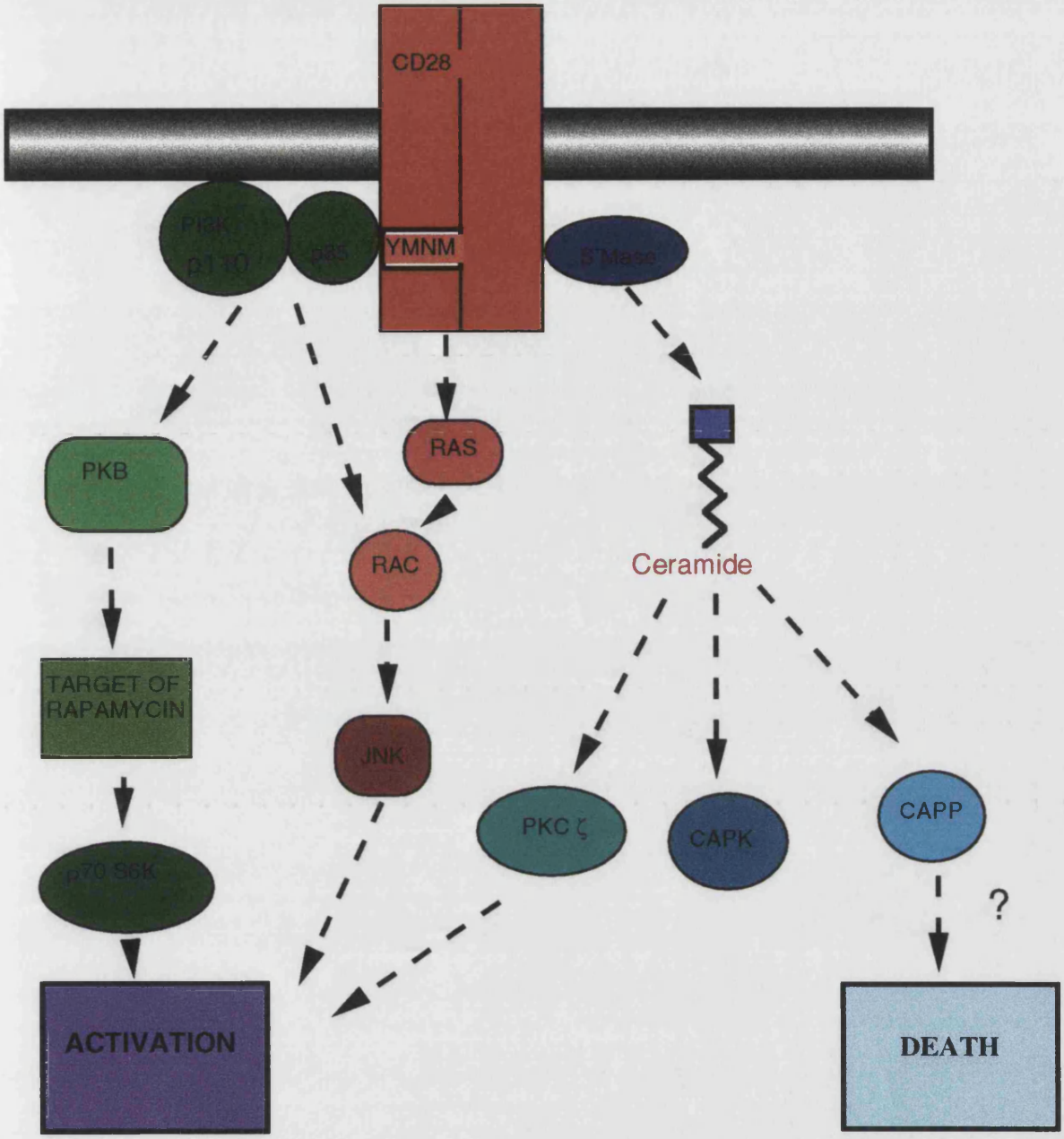


Figure 1.4 A simplified diagramatic representation of the current understanding of the main CD28 signalling pathways

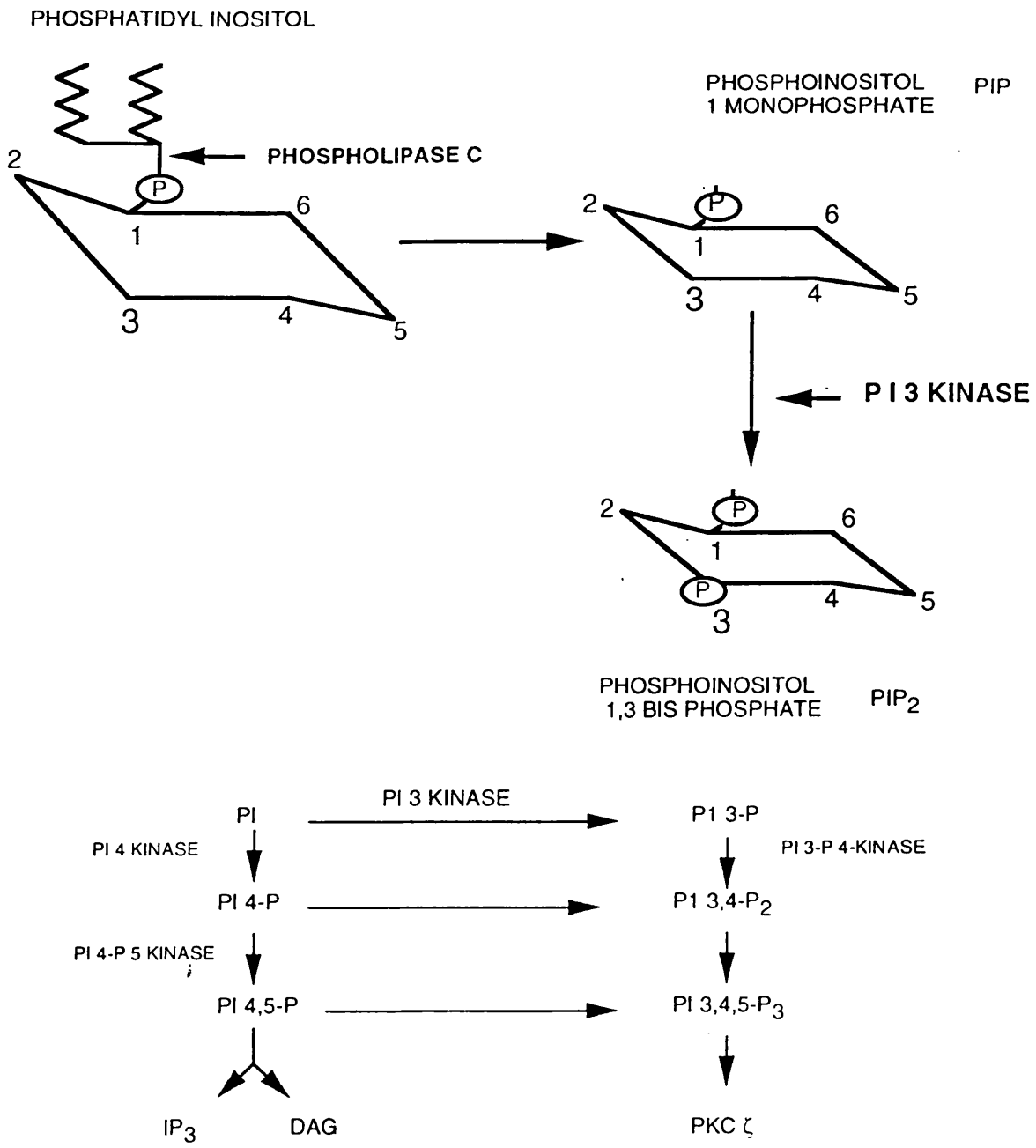


Figure 1.5 A diagramatical representation of the structure and different phosphorylated forms of the phosphoinositide molecules associated with PI3 Kinase signalling activity

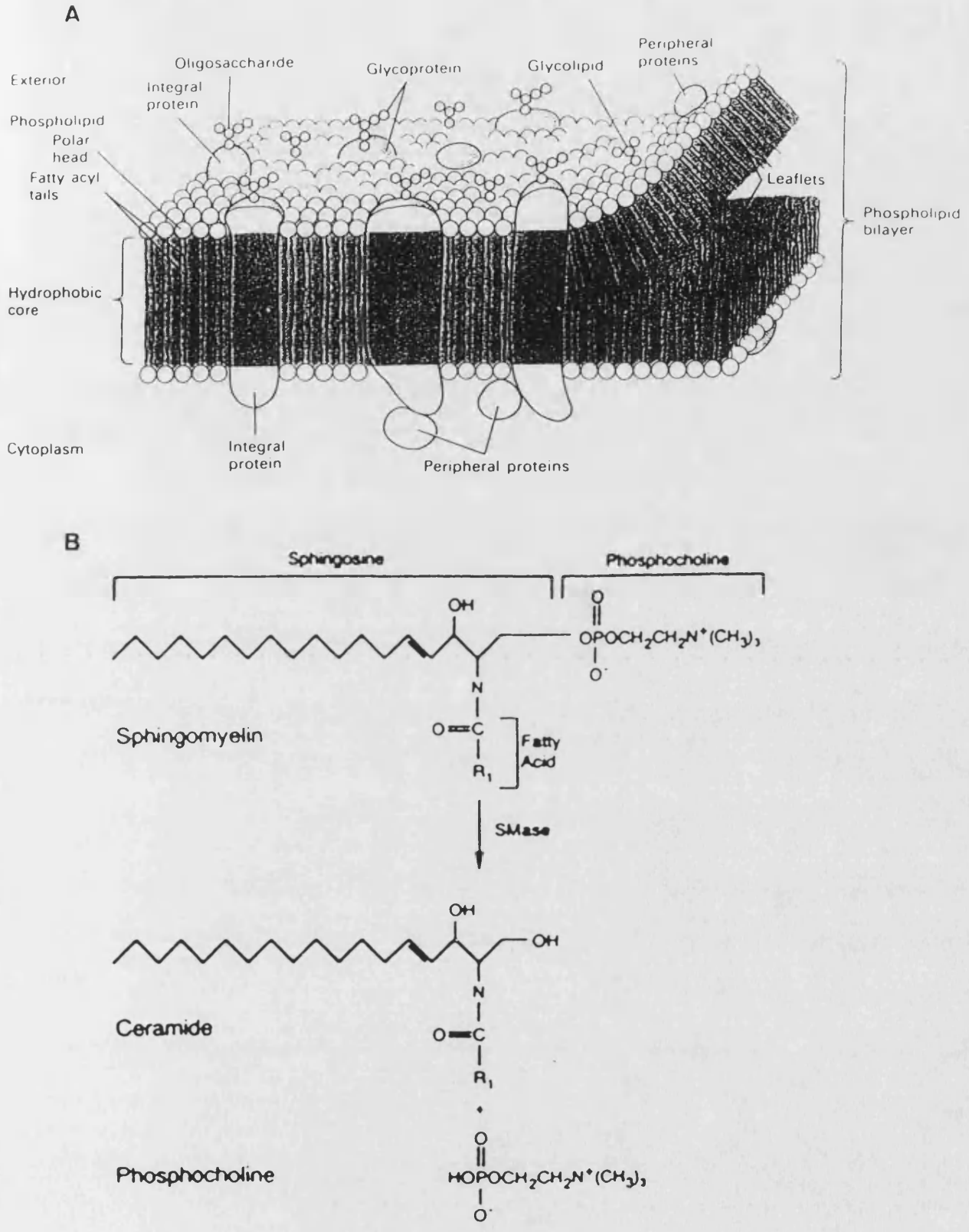


Figure 1.6 (A) A general model of the structure of biological membranes (Molecular Cell Biology. Darnell,J., Lodish,H. and Baltimore,D. page 571).(B) Sphingomyelin hydrolysis by sphingomyelinase (The Sphingomyelin Pathway in Tumour Necrosis Factor and Interleukin-1 Signalling. Kolesnick,R. and Golde,D.W. (1994) Cell 77:325)

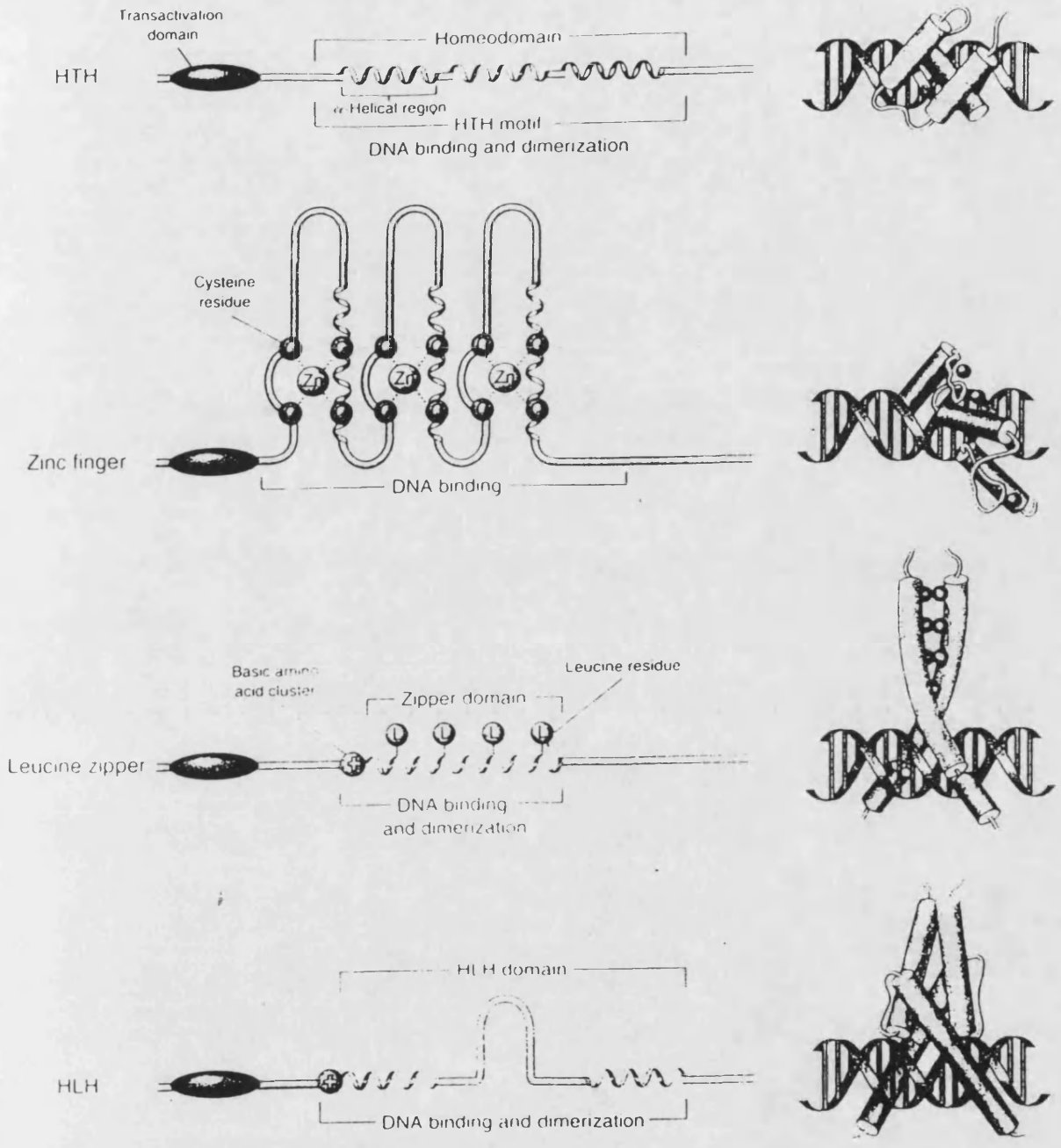


Figure 1.7 The four different structural forms of transcription factor protein complexes (reproduced from ' Transcription Factors' {Papavassilou})

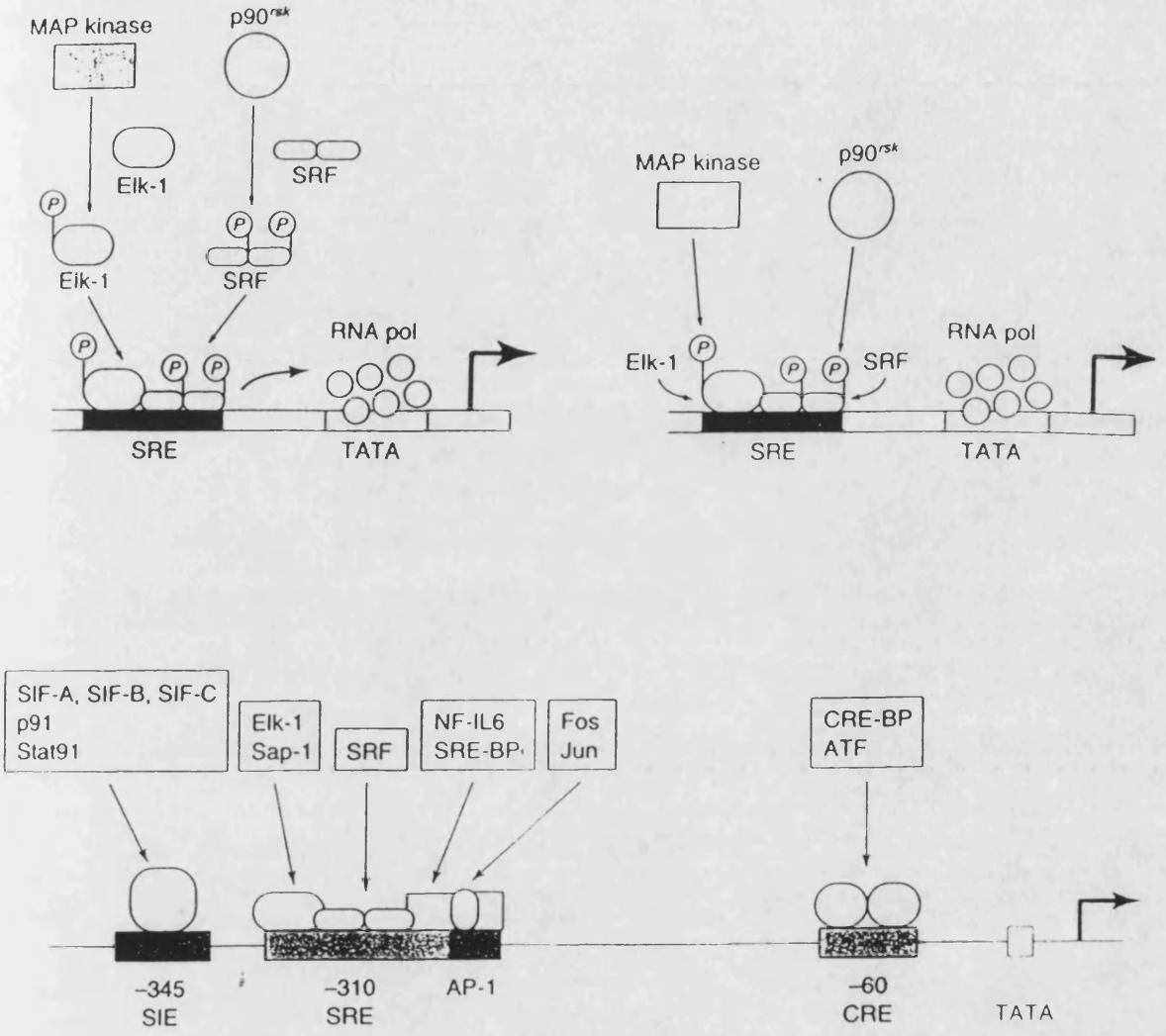


Figure 1.8 A diagramatic representation of the promoter region of the c-fos gene showing the known regulatory binding sites (reproduced from a review by {Edwards})

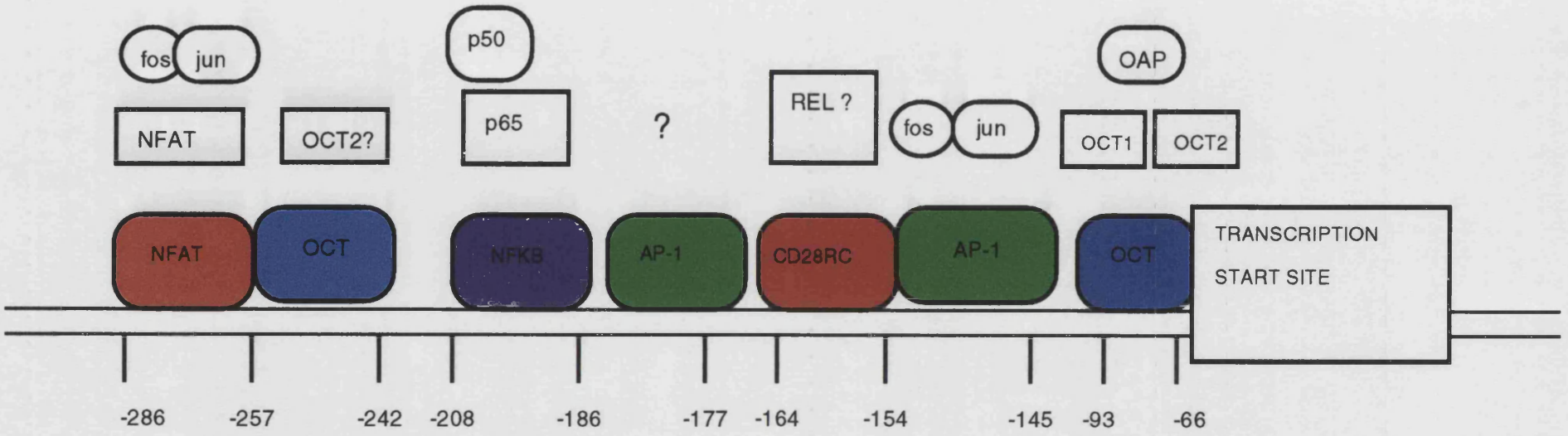


Figure 1.9 The Interleukin-2 promoter region showing the location and composition of the known regulatory transcription factors

CHAPTER TWO - METHODS

2.0 Reagents

Unless otherwise specified, all laboratory reagents were purchased from Sigma with the exception of the following which were kindly donated from various sources. The Jurkat cells and Quantikine cytokine assay kits (R&D Systems Cat. Nos. IL-2 D2050, IL-4 D4000) were donated by Dr. A. Lennard at the Yamanouchi Research Institute in Oxford whilst the CTLLs and the recombinant IL-2 standard were obtained from the National Institute of Biological Standards and Controls (NIBSC). The IL-2 which was used to culture the T cell blasts was obtained from Glaxo, the staphylococcal enterotoxin A (SEA) was a gift from M. Dohlsten, Pharmacia, Sweden and the GST-jun fusion protein was supplied by J. Woodget, Toronto. The antibodies used in the experiments were obtained from a variety of sources. OKT3, OKT11, HB8784 and L243 were all obtained from ATCC, E2H10, UCHT4 and UCHM1 were donated by Prof. P. Beverley, Dr. P. Linsley supplied the 9.3 and BB-1 antibodies and Dr. I. McLennan donated the anti CD19. Finally the PKC inhibitor Ro 31-8220 was a gift to the pharmacology department of the University from Roche Pharmaceuticals.

2.1 Cell culture

Assays were carried out using the leukaemic T cell line Jurkat, peripheral blood T cell blasts and purified resting T cells, the latter two of which were prepared from blood samples from normal donors. Both the T cells and the Jurkat cells were cultured in 175cm^2 tissue culture flasks at concentrations of 1.5-3x10^6/ml in RPMI 1640 containing 10% FCS supplemented with penicillin and streptomycin (see appendix 1).

The transfected cell lines were produced in our laboratory as previously described (Sansom et al., 1993). Briefly CHO cells were transfected with human cDNA's encoding various antigens including B7-1 (CD80) and HLA-DR4 using the pEE6 expression vector under the control of a CMV promoter (see appendix 5). Positive clones were selected and expression levels of the relevant antigen monitored regularly using FACS analysis of surface staining.

All the transfectant cells were cultured in Glutamine -Free DMEM containing 10% FCS supplemented with nucleosides, sodium pyruvate and antibiotics as above (see appendix 1). This media was used to allow for selection of surface expression under the regulation of glutamine synthase. The cells were passaged every three days by aspiration of the media followed by a PBS wash and a 5 minute incubation with trypsin at 37°C. The cells were then dislodged by gentle tapping of the flask. Following the addition of media to

inactivate the trypsin, 90% of the cells were removed and the remaining diluted to volume with fresh media.

2.2 FACS Analysis

FACS analysis of the cells being used for experimental purposes provides a relative quantitation of constitutive and inducible cell surface antigens and can give an indication of how stimuli may affect a particular cell type or help to explain why cells respond in a particular way to a certain treatment. The analysis involves labelling of surface markers, using specific monoclonal antibodies followed by detection using a fluorescent labelled antibody which targets the primary antibody. FACS analysis of resting T cells or T cell blasts prior to their use in an experiment also provided quality control information on cells obtained from different donors.

Typically $5x10^5$ cells were stained for relevant surface markers using standard mAbs, OKT11 (αCD2), OKT3 (αCD3), E2H10 (αCD4), UCHT4 (αCD8), HB8784 (αCD25), 9.3 (αCD28), BB-1(αB7-1) and L243 (αHLA-DR) whilst cells in medium were used as a negative control. Supernatants, obtained from hybridoma cell lines, contained antibody concentrations of approximately 1μg/ml. 50μl of the hybridoma supernatant were added to the cell suspension and incubated at 4°C for 45minutes. Excess or unbound primary antibody was removed by washing the cells in PBS. Cells were recovered by centrifugation at 1500rpm for 5 minutes (Beckman GPR) and primary antibodies were detected using polyvalent α mouse FITC incubated as above. After washing the cells were resuspended in 1ml of PBS and analysis was carried out on a Becton Dickinson FACStar at 488nm using a 60mW laser. 5000 events were typically recorded as a representative sample.

2.3 Preparation of T cell blasts from peripheral blood

Due to the large numbers of cells required for some of the assays it was not possible to obtain sufficient resting T cells from blood donations. An alternative option was to expand cell numbers by the production of T cell blasts which would proliferate to exogenous stimuli and which could then be quiesced to give a more physiological model of T cell activation. A standard protocol, detailed below, was adopted for the production of T cell blasts to minimise differences between cultures.

50 - 100mls of blood were collected into heparinised tubes (0.1%$^{v}/_{v}$ of 5000U/ml, 'Monoparin' CP pharmaceuticals). The blood was diluted 1:1 with PBS, layered onto a lymphoprep gradient (density 1.077g/ml, Nycomed) and centrifuged without a brake at 1500rpm for 30min (Mistral 2L). The serum was aspirated off and the buff interface was

collected. These mononuclear cells were then washed three times with RPMI 1640 containing 10% FCS. The cells were counted using a Neubauer haemocytometer and the volume adjusted to give a concentration of $5x10^6$ cells/ml. The cells were placed in a tissue culture flask and stimulated with the phorbol ester, PMA (50ng/ml) and the calcium ionophore, ionomycin (1μM) (Calbiochem Cat. No. 407950). The cells were cultured for 8 days with media being added as required. After this period the cells were washed twice in RPMI 1640 with $10\%^{v}/_{v}$ FCS and were then used for the various experiments. To generate staphylococcal enterotoxin A (SEA)-responsive blasts PBMCs were prepared as described above and soluble SEA was added at a concentration of 10ng/ml. After 8-10 days the blasts were restimulated with SEA antigen pulsed onto DR4/B7 transfectants which acted as APCs.

To bind the antigen to the transfectants, SEA (10ng/ml) was added to DR4/B7 transfectants ($5x10^6$/ml) and incubated at 37°C for 4 hours with occasional gentle mixing. The cells were then washed twice and fixed in glutaraldehyde as described below. The transfectants were added to the blast cultures at a ratio of 1: 5 T cells which were then left a further 4-10 days with the addition of RPMI 1640 media containing 10% FCS as required. The media was supplemented with IL-2 (100U/ml recombinant human IL-2) every 4 days.

2.4 Preparation of purified T cells

Purified resting T cells were prepared from blood samples initially using the same method as described above. However, after counting, the PBMCs were placed in petri dishes and were incubated at 37°C for 1 hour to remove adherent cells such as monocytes. The non-adherent cells were carefully removed and washed twice in media. The final pellet obtained after washing was resuspended in 500μl each of L243 and CD14 antibody supernatants and 10μl of CD19 ascites to label any remaining MHC class II DR4 positive cells, monocytes and B cells respectively. The cells were placed on a rotator at 14°C for an hour and were then washed again. The recovered pellet was resuspended in 500μl of media and 50μl of immunomagnetic sheep anti-mouse IgG beads before another incubation period on the rotator. The cells were diluted out in media and applied to a magnet three times to remove the bead-bound cells. The remaining cells were washed, counted and FACS analysed. Typical preparations were >95% $CD3^+$.

2.5 Proliferation assays

Proliferation assays enable the establishment of a response profile showing the general functional effects of different stimulation conditions on different cell types. Thymidine is required by proliferating cells during DNA synthesis and its rate of uptake into a cell is

proportional to the amount of proliferation. This relatively sensitive assay measures changes in cell proliferation by recording ^{3}H-Thymidine incorporation. In the assay, cells are incubated in the presence of various reagents to allow the effects on proliferation to occur. The cells are then supplied with ^{3}H-Thymidine and later, after a further incubation, analysed to assess the amount of Thymidine incorporated. By comparing the levels of radioactivity in stimulated and unstimulated cells an assessment can be made of the effect of the stimulus.

In order to prevent high background counts due to CHO/transfectant proliferation these cells were fixed prior to use. This effectively kills the cell stopping all further growth and intracellular reactions but leaves the cell whole, enabling its surface molecules to interact with corresponding ligands/ receptors on other cells. To fix the transfectants 2-5x10^{6} cells were trypsinised, washed twice in PBS and then resuspended for 2 min in 1ml 0.025% $^{v}/_{v}$ glutaraldehyde. The cells were vortexed to ensure complete resuspension and then diluted with media. After centrifugation at 1500rpm for 5 minutes the cells were washed twice in media and resuspended at the required concentration for the assay. Control wells of fixed transfectants alone were included in the assay to ensure the fixation procedure was complete and that any proliferation could not be attributed to the transfectants themselves.

In stimulation assays 5x10^{4} T cell blasts and purified resting T cells were aliquotted into round-bottomed 96-well plates. Fixed transfectants were added at 2x10^{4} cells per well and the other stimuli used at the concentrations stated in individual experiments. Monoclonal antibodies were added in soluble form in combination with a sheep anti-mouse IgG cross-linking agent (Sigma M1522) unless otherwise stated and each treatment was carried out in triplicate.

The assays were incubated at 37°C for the appropriate length of time. 50µl of supernatant were then removed from each well and assayed for IL-2 production as detailed below. 1µCi of ^{3}H-Thymidine (6.7 Ci/mmol, ICN Cat No.2406605) was added to each of the wells and the plate was returned to the incubator for a further 8 hours. After this time the cells were harvested onto glass fibre filters, separated into tubes containing 0.5mls scintillation fluid (Wallac ' Optiscint HiSafe') and the radioactivity was measured using a β liquid scintillation counter.

Proliferation assays were carried out on the Jurkat T cell line as described above except that the cells were seeded at an initial concentration of 5x10^{3} Jurkat cells / well. All other conditions were identical to those for the T cell blasts.

2.6 Cytokine analysis

IL-2 production by stimulated cells was routinely measured using a bioassay based on the proliferation of an IL-2 dependent cell line, CTLLs. Some IL-2 and IL-4 assays, however, were carried out using ELISA kits and were performed according to the manufacturers instructions.

2.6.1 CTLL IL-2 Bioassay

Murine CTLL cells undergo IL-2-dependent growth and if calibrated can be used to measure IL-2 concentrations in sample supernatants. CTLL cells were cultured in RPMI 1640 containing 10% ($^{v}/_{v}$) FCS, as for the peripheral blood T cells (see appendix 1) and fed with human recombinant IL-2 at 10-15 U/ml on a three day cycle. When required, the cells were passaged before being fed IL-2. The viability of the cells was checked using trypan blue exclusion and was routinely greater than 90%.

An IL-2 standard curve was produced by two-fold serial dilutions of an IL-2 standard in the range of 0.019 - 20 U/ml. $5x10^3$ CTLL cells were added to each well of a round-bottomed 96 -well plate containing 50µl of IL-2 standard or a 1:10 dilution of sample supernatant. Both samples and standards were assayed in triplicate. The plate was incubated overnight, 0.5µCi of ^3H Thymidine were added to each well and then the assay was incubated for a further 4 hours before the cells were harvested onto glass fibre filters. A calibration curve was plotted from the standards and the concentration of IL-2 in the samples was calculated.

2.6.2 ELISA assays

The enzyme linked immunosorbant assay (ELISA) is a relatively rapid and sensitive assay for the detection of soluble proteins in cell supernatants and works on the principle of detection of specific proteins by antibody binding.

Briefly supernatant samples taken from the proliferation assays were aliquotted into 96-well plates coated with a specific antibody to the protein of interest. After a period of incubation to allow binding, excess supernatant was removed and specific binding was detected using a second antibody also directed against the protein. This was linked to an enzyme which in the presence of added substrate caused a rapid colour change which could be rated visually or by spectrophotometry. IL-2 and IL-4 standards were used to calibrate the assays which were analysed on a plate reader at 450nm (Dynatech MR5000).

2.7 Analysis of the effect of PMA stimulation on Jurkat cells

Results obtained from the proliferation assays indicated that whilst PMA in the presence of ionomycin induced proliferation in normal T cells this stimulus had an inhibitory effect on Jurkat cell proliferation. Due to this unexpected observation additional experiments were undertaken to further investigate these effects. Three assays described below were performed to assess the effects of PMA on Jurkat cell cycle and viability.

2.7.1 Cell viability

Jurkat cell suspensions of $1x10^6$/ml were aliquotted into 24-well plates and either left unstimulated or treated with a combination of PMA (30ng/ml) and ionomycin (1μM). The cells were incubated at 37°C for 72 hours after which period an aliquot was removed and mixed 1:1 with Trypan blue dye. Cell number and viability were then assessed visually with the aid of a Neubauer haemocytometer.

2.7.2 Propidium iodide staining of nuclei to assess apoptotic cell death

Cytospins were prepared from a second aliquot of the cells. Once dry the slides were fixed in acetone for 5 minutes and allowed to air dry. Propidium iodide(PI) solution was added in excess for 30 seconds and then removed by washing the slides with PBS. Mountant (95% glycerol, 2.5% 1,4 Diazabicyclo 2,2,2 octane in PBS) was immediately added and a coverslip placed over the sample. Cellular staining was assessed visually using a fluorescent microscope and comparisons between the stimulated and unstimulated cells were made.

2.7.3 Cell cycle analysis

In addition the effect of PMA stimulation on Jurkat cell cycle was assessed. $1x10^6$ cells were pelleted by centrifugation at 1500rpm for 5minutes (Beckman GPR). The pellet was resuspended in 500μl of ice cold 80% ethanol in PBS and the cells were incubated at 4°C for 2 hours. Cell pellets were obtained as before and were resuspended in PBS containing RNase (20μg/ml). The cells were incubated for 30 minutes at 37°C prior to the addition of PI (4μg/ml). After a further 15 minute incubation at room temperature the cells were centrifuged and resuspended in PBS before being analysed for PI fluorescence at 580nm by FACS using a 60mW laser.

2.8 Electromobility gel shift assay (EMSA)

EMSA allow the detection of specific DNA-binding proteins (transcription factors) in extracts produced from resting and stimulated cells. Nuclear extracts, prepared from cell cultures, are incubated with radiolabelled oligonucleotides which are short sections of double stranded DNA, the sequence of which defines a binding site for the relevant transcription factor. Bound and free oligonucleotide can be separated on a non-denaturing polyacrylamide gel due to retardation of the protein/ DNA complexes and can then easily be visualised by autoradiography.

2.8.1 Preparation of the nuclear extracts

Cells (approximately $6x10^7$) were taken from culture, washed twice in medium, counted, resuspended at a concentration of $3\text{-}5x10^6$/ml and replaced in 50ml plastic tubes. Stimuli were added at the specified concentrations and when required glutaraldehyde fixed transfectants were added a ratio of 1: 5 T cells.

Following a 4 hour incubation at 37°C with occasional gentle mixing, the cells were washed in cold PBS. The pellet was resuspended in 400µl of hypotonic buffer 1 with 0.2% NP40 (10mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.2mM PMSF, 0.7mM pepstatin, 1mM E64 and 0.5mM leupeptin) transferred to an eppendorf and left on ice for 15 minutes. Nuclei were pelleted by centrifugation at 2000rpm in a bench top microfuge (Beckman GS15R) for 10 minutes and the supernatant containing the cytoplasmic extract was retained for functional assays and stored at -80°C until required.

The nuclear pellet was gently washed in 800µl of buffer 1 containing 8.5% sucrose and recovered by centrifugation as above. The clean nuclei were gently resuspended by sequentially adding 100µl aliquots of hypertonic buffer 2 (20mM Hepes pH 7.9, 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and protease inhibitors as above) to a final volume of 400µl and incubated on ice for 20 minutes during which period the nuclei lysed releasing the proteins into the supernatant. Nuclear membranes and DNA were removed by centrifugation at 14000rpm for 30 minutes. Ammonium sulphate (0.33g/ml) was added to the supernatant to a final concentration of 3M to precipitate the proteins which were then pelleted by centrifugation as described above. The protein pellet was finally resuspended in 50µl of buffer 3 (20mM Hepes pH 7.9, 20mM KCl, 1mM $MgCl_2$, 2mM DTT, 17% glycerol and 0.2mM PMSF) and assayed for protein content using the Biorad dye reagent as described below. Aliquots of the extract were flash frozen in liquid nitrogen and stored at -80°C until required.

2.8.2 Biorad protein assay

The protein content of the nuclear extracts was assessed using Biorad dye reagent. This is a colourimetric assay based on the Bradford dye- binding procedure which measures the colour change observed following the binding of Coomassie brilliant blue G-250 dye to the basic and aromatic amino acid residues of proteins. The assay was calibrated using a standard curve but since purified transcription factor protein was not available, BSA was used as a relative standard.

A calibration curve of 0-10μg/ml of BSA standard was constructed by serial dilution in PBS and the samples were prepared by adding 1μl of extract to 800μl of PBS. 200μl of dye reagent (55% phosphoric acid, 15% methanol; Biorad, Cat. No. 500 0006) were added to 800μl of each standard and sample. After a 5 minute incubation period at room temperature the samples were assayed by UV / visible spectrophotometry at 595nm (LKB biochrom Ultrospec II). A calibration curve was plotted and the concentration of each of the extracts calculated.

2.8.3 Labelling of the oligonucleotides

Oligonucleotides, typically between 15 and 30 bases, representing the sequence of the appropriate transcription factor binding site, were synthesized as two complementary single strands. One oligonucleotide strand was radiolabelled by the transfer of a γ-^{32}P molecule from γ-^{32}P-ATP by the bacterial enzyme T4 polynucleotide kinase prior to the annealing of the complementary strands. This reaction occurs independently of oligo sequence and can also be employed in the labelling of double stranded oligonucleotides. The DNA sequences of the oligonucleotides used are shown below. The AP-1 and NFAT oligonucleotides were a kind gift from Dr. A. Lennard at the Yamanouchi Research Institute in Oxford whilst the NFκB oligonucleotide was purchased from Promega (Cat. No. E3291)

AP-1	5' CGC AAG TGA CTC AGC GCG 3'
	3' GCG TTC ACT GAG TCG CGC 5'
NFAT	5' GGA GGA AAA ACT GTT TCA TAC AGA AGG CGT 3'
	3' CCT CCT TTT TGA CAA AGT ATG TCT TCC GCA 5'
NFκB	5' AGT TGA GGG GAC TTT CCC AGG C 3'
	3' TCA ACT CCC CTG AAA GGG TCC G 5'

A labelling reaction containing 50ng of single stranded oligonucleotide, 25μCi ^{32}P γ-ATP (4500 Ci/mmol, ICN Cat. No. 38101X), PNK buffer and enzyme (Sequitherm kit, Epicentre Technologies) in a final volume of 5μl was incubated at 37°C for 40 minutes. The reaction was stopped by heating to 70°C for a further 10 minutes to deactivate the enzyme. 50ng of the complementary strand were added and the reaction diluted to 10μl with TE buffer (see appendix 2). The two strands were annealed by heating to 65°C for 5 minutes, followed by incubation at 37°C for 5 minutes. The samples were then cooled to room temperature before being diluted to 100μl with TE buffer and placed on ice. Unincorporated label was removed by passage of the oligonucleotide solution through a Sephadex G50 spin column (Pharmacia Cat. No. 17-0862-01) equilibrated with TE buffer. An aliquot of the eluate was taken prior to each experiment and a sample of this was counted for radioactivity. The aliquot was then diluted to give 20000cpm / μl ready for addition to the binding mix.

2.8.4 EMSA

Typically 15μg of nuclear extract in 12μl of buffer 3 were added to a binding mix containing 1μg poly dI-dC (Pharmacia Cat. No. 27-7880), 7.5% $^{v}/_{v}$ glycerol, 38mM KCl and 0.6mM $MgCl_2$. After a 10 minute preincubation period radiolabelled oligonucleotide was added (40000cpm in 2μl) to give a final volume of 20μl. The samples were incubated for 20 minutes at room temperature before loading onto a 5% $^{v}/_{v}$ native (non denaturing) polyacrylamide gel (see appendix 3). 5μl of 6% $^{w}/_{v}$ loading dye (see appendix 2) was added to a no-extract control as a marker during the running of the gel. The gel was run in 0.5x TBE (see appendix 2) at a constant voltage of 150V for a period of approximately two hours. On completion the gel was fixed in 10%$^{v}/_{v}$ MeOH, 10%$^{v}/_{v}$ glacial acetic acid and 5%$^{v}/_{v}$ glycerol prior to drying onto filter paper and autoradiography. The duration of the exposure of the film varied depending upon the relative abundance of the protein and the activity of the oligonucleotide but was typically 24 hours.

2.9 Sphingomyelinase assay

Several signalling pathways in T cells, including that of CD28, have been proposed to involve the activation of the phospholipase enzyme sphingomyelinase. This, when activated, hydrolyses the sphingosine-based membrane lipid, sphingomyelin to produce ceramide and phosphocholine. This assay, based on the method described by Boucher et al (1995), enables the detection of sphingomyelinase activity by the measurement of phosphocholine levels in aqueous cell extracts. Stimulated cells are incubated with ^{14}C sphingomyelin and the radioactive products are detected by chromatographic or scintillation methods. The latter method was used here to assess sphingomyelinase activation in cells taken from cultures used for the EMSA.

T cell blasts or Jurkat cells were incubated in serum- free RPMI 1640 medium at 37°C for a minimum of two hours to ensure against serum induced enzyme activation. $3x10^6$ cells in 100µl aliquots were then transferred into individual eppendorfs and the appropriate stimuli were added. All the transfectants used were glutaraldehyde fixed and washed twice prior to mixing. At the relevant time points the reaction was stopped by immersion of the eppendorf in liquid nitrogen, followed by dilution of the sample with 1ml of ice cold PBS and centrifugation for 10-15 seconds at 2000rpm using a refrigerated microfuge (Heraeus Biofuge Fresco). Cell pellets were washed twice in cold PBS, once in buffer A (20mM Tris- HCl pH 7.4, 137mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$ and protease inhibitors as for EMSA buffer 1) and were recovered by centrifugation as above. The final pellet was incubated on ice in 200µl of buffer A containing 0.5% NP40 for 15 minutes. Supernatants were recovered following centrifugation at 13000rpm for 5 minutes and assayed for protein content using the Biorad dye method detailed above. 30-50µg of protein were diluted in buffer B (250mM NaAc, 1mM EDTA (pH5), 1.1µCi/ml ^{14}C-Sphingomyelin (Amersham CFA566)) to a final volume of 50µl. After incubation for 2 hours at 37°C the samples underwent chloroform : methanol extraction (2:1). Briefly 800µl of the chloroform/ methanol mix were added to the sample which was diluted to a final volume of 1.1mls with H_2O. After vortexing and high speed centrifugation as above 200µl of the aqueous phase were mixed with 5mls of scintillation fluid and counted. A timecourse of enzyme induction was plotted as percentage increase above basal levels.

For experiments involving enzyme inhibitors the cells were preincubated for 1 hour with appropriate concentrations of the inhibitor or PBS vehicle after the incubation in serum-free medium.

2.10 JNK assays

JNK activation can be detected *in vitro* as a phosphorylation of the JNK substrate, c- jun. In this assay, based on the method described by Derijard et al (1994), lysates from stimulated cells are incubated *in vitro* with the fusion protein GST-jun comprising amino acids 5-89 of the N terminus of c-jun. Addition of radioactive phosphate enables the phosphorylation of the GST-jun by activated enzyme which can then be detected by gel autoradiography.

$5x10^6$ Jurkat cells or T cell blasts were stimulated for a period of time (1-30 minutes) and were then lysed in buffer A with 0.2% NP40 as described above (sphingomyelinase assay). The 1ml lysate was incubated overnight on a rotator at 4°C with GST-jun coated agarose beads. The beads were pelleted by gentle centrifugation and were washed once in lysis buffer and once in kinase buffer (25mM Hepes pH7.4, 100mM NaCl, 5mM $MnCl_2$, 10mM $MgCl_2$, 100µM Sodium orthovanadate and 10µM Mg^{2+} ATP). The

kinase buffer was aspirated from the bead pellet which was then resuspended in a further 30µl of kinase buffer at room temperature. To this were added 10µCi of ^{32}P γ-ATP (4500 Ci/mmol, ICN Cat. No. 38101X) and the samples were left for 30 minutes. The reaction was stopped by two washes in kinase buffer containing 30 mM EDTA followed by draining of the pellet and its resuspension in 10µl of sample buffer (see appendix 2). The samples were boiled for 10 minutes and run on a 10% SDS-PAGE gel (see appendices 2 and 3). Protein was visualised by coomassie staining before the dried gel was autoradiographed. Results are shown as an image captured using Imagedok analysis program software.

After optimisation of the assay in Jurkat cells using U.V stimulation, the B7-stimulated T cell blast cytoplasmic extracts produced as by-products of the EMSA were analysed for JNK activity as a comparison between the ability of stimuli to induce transcription factor and JNK activity.

2.11 DNase 1 footprinting assays

A DNAse footprint assay is based on a similar principle to that of the EMSA. Cells are stimulated to produce transcription factor proteins which bind a large specific radiolabelled probe usually coding the whole gene promoter of interest. The DNA is then degraded using a DNase digestion enzyme. However, the regions of DNA bound by protein are protected from DNase digestion and can be gel analysed to reveal their position within the gene promoter. This enables a better assessment of physiological binding to gene promoters as it allows competition between factors for binding sites as well as cooperative binding. Experiments were carried out to assess the protected regions of the IL-2 promoter with the aim of studying the binding characteristics of the CD28RC.

The footprint assay required the preparation of nuclear extracts as detailed above and the generation of both an oligonucleotide probe and a G+A sequencing marker the preparation of which is based on the Maxam and Gilbert sequencing method (Sambrook et al., 1989) and which is described briefly below.

2.11.1 Plasmid preparation

A PCR product of the IL-2 promoter region was generated at the Yamanouchi Research Institute at Littlemore Hospital Oxford and kindly donated. This was subcloned directly into the mammalian TA cloning vector, pCR3 (see appendix 5) by ligation of the insert into the vector which was carried out overnight at 14°C with unligated and religated controls. Competent E. Coli Top 10F cells were transformed with the ligations by heat shock using an Invitrogen eukaryotic TA cloning kit (Cat No. K3001-01). Briefly 2µl

0.5M β-mercaptoethanol were added to a 50µl aliquot of Top 10F cells and mixed by gentle stirring. 2µl of ligation mix were then added and the samples incubated on ice for 30 minutes. The samples were placed in a water bath at 42°C for exactly 30 seconds and then returned immediately to ice for a further 2 minutes. 800µl of SOC media (see appendix 4) were added to each vial which were then placed in an orbital shaker at 37°C for 1 hour. Aliquots were then plated out on ampicillin-containing agar plates (see appendix 4) and left at 37°C overnight.

Several bacterial colonies were picked at random from the agar plates and grown overnight in 5ml broth minicultures (see appendix 4). Minipreps were prepared from each using the standard alkaline lysis protocols by Birnboim and Doly and Ish-Horowicz and Burke which are described in Maniatis (Sambrook et al., 1989). Single and double restriction enzyme digests were carried out and analysed on a 1x TAE buffered 1% agarose/ ethidium bromide gel (see appendices 2 and 3) to isolate cultures carrying the IL-2 promoter insert. A 500ml maxiprep was prepared from one positive culture using the above methods and the plasmid prep was PEG purified (R. Treisman, Maniatis (Sambrook et al., 1989)). The pellet was resuspended in TE buffer and the DNA concentration was assessed by spectrophotometry at 260nm.

2.11.2 Footprinting Probe

30µg of the plasmid were cut to completion with a restriction enzyme approximately 100bp from the site of interest to leave a 5' overhang. Excess calf intestinal phosphatase (CIP) (Boehringer Mannheim Cat. No. 713023) was then added to the reaction mix which was incubated at 37°C overnight to ensure complete removal of the 5' terminal phosphatases. The CIP was removed by one phenol only and one phenol/ chloroform extraction followed by ethanol precipitation. The recovered linear plasmid was resuspended in TE at 2.5µg/µl and a 5µg aliquot was removed for 5' end labelling. The labelling reaction was carried out as described for the EMSA oligonucleotide but before purification the plasmid was cut for a second time using excess of a restriction enzyme cutting at a site 60-700 base pairs from the first.

The probe was purified by adding loading dye (see appendix 2) directly to the digest mix and running the sample on a 5% non denaturing acrylamide gel (see appendix 3) in 1xTBE (see appendix 2) at 150V for approximately 2 hours. The gel was exposed to X-ray film for 2 minutes and the required band was located and excised with the aid of fluorescent markers to help alignment of the film and gel. The probe was electroeluted from the gel slice in 0.5xTBE at 100V for 1 hour into a high salt solution. Purified probe was recovered by phenol/chloroform extraction and ethanol precipitation in the presence

of excess glycogen to act as a carrier. The probe was stored at -20°C until required when activity was assessed by β emission counting.

2.11.3 G+A Sequencing Ladder

200000cpm of probe was diluted to 20µl with H_2O and placed on ice for 5 minutes. 50µl of Formic acid were added and the probe incubated at 20°C for 3.5 minutes. The reaction was stopped with 180µl of HZ stop solution followed immediately with 750µl of ethanol. The probe was recovered by precipitation on dry ice for 10 minutes followed by centrifugation at 14000rpm for 20 minutes in a microfuge. The pellet was resuspended in 0.3M NaAc and reprecipitated with ethanol. The recovered pellet was washed with ethanol and dried before resuspension in 1M piperidine and incubation at 90°C for 30 minutes. The pellet was lyophilised for 4 hours, resuspended in water and lyophilised again overnight. After a third lyophilisation the pellet was resuspended in water and the activity assessed by β emission counting. An aliquot of marker corresponding to 7000cpm was then taken to which 5µl of formamide blue were added in preparation for gel loading.

2.11.4 DNase 1 Digestions

The initial assay was to determine an appropriate concentration of DNase enzyme to obtain optimal digestion. Two sets of eppendorf tubes with and without cell extracts were set up covering a range of DNase enzyme concentrations as lower concentrations were required in the absence of extract. 12µg of extract in a final volume of 20µl ,were incubated at room temperature for 10 minutes in the presence of salt mix (KCl, $MgCl_2$), extract buffer and poly dI-dC (1µg). 2µl of probe representing 20000cpm were then added and the mix preincubated at 20°C for 20 minutes to allow protein binding to reach equilibrium. 3µl of the appropriate DNase1 solution was then added to the mix followed exactly 1.5 minutes later by 200µl of stop solution. The digestions were recovered by phenol/ chloroform extraction followed by ethanol precipitation. The pellets were resuspended in 5µl formamide blue and both the samples and G + A marker were boiled for 2 minutes.

2.11.5 Gel

The samples were loaded onto a 6% gradient sequencing gel (see appendix 3) and run in 1xTBE at 1000V/ 50mA for approximately five hours until the cyanol blue had reached the bottom of the plate. The gel was removed and fixed for 20 minutes in 10%methanol / 10% acetic acid prior to drying and autoradiography . The G+A ladder was aligned with the IL-2 promoter sequence enabling location of the protected regions.

Several experiments were carried out initially in order to optimise the conditions required including titrations for DNase, poly dI-dC and $MgCl_2$. Extracts produced from stimulated and unstimulated cell cultures were then tested to obtain appropriate footprints.

2.12 Reporter construct assays

Reporter construct assays have proved extremely useful as laboratory tools especially for the study of the regulation of gene promoters by transcription factors. Two, well characterised protocols involve the cellular transfection of transcription factor driven CAT (Gorman et al., 1982; Sleigh, 1986) or luciferase reporter constructs. Plasmids containing either a constitutive promoter or multiple transcription factor binding sites upstream of the enzyme reporter gene are transfected into a host cell (see appendix 5). The transfections can be achieved in a variety of ways although the methods detailed here involve the technique of electroporation. After a recovery and stimulation period if required, the cells are lysed to produce cytoplasmic extracts containing the transcribed enzyme. An *in vitro* assay then enables measurement of enzymatic activity which can be correlated with the activation of the cell by the stimulus or in the case of constitutive activity, acts as a positive control. The aim of these assays was to assess the ability of certain stimuli to induce functional transcription factor complexes. Whilst the EMSA detects the induction of a transcription factor it does not show that this is functional transcriptionally. Initially constitutive CAT assays were carried out to enable optimisation of the electroporation conditions required for the transfection of the cells but these were later replaced by luciferase assays due to the reported increased sensitivity of the latter (Alam and Cook, 1990).

2.12.1 Electroporation of Jurkat cells

Initial electroporation conditions were obtained from the literature and were then optimised for the Jurkat cells in terms of voltage, time constant and electroporation buffer to give a maximal permeabilisation to viability ratio. Comparisons between different conditions were made using an FDA/ PI FACS analysis assay.

Jurkat cells were washed and resuspended in either HeBS or Ca^{2+}/ Mg^{2+} PBS buffer at a concentration of $5x10^6$/ml and were electroporated over a range of voltages (200-400V) at varying capacitances (250-960μF). A 200μl aliquot of electroporated cells were placed on ice and incubated for 10 minutes with 4μg/ml PI. The cells were then diluted in 1ml of culture medium and incubated at 37°C for 30 minutes. Following this they were washed in PBS and resuspended in 1ml of PBS containing 0.1ng/ml FDA solution. FACS analysis of the cells produced a single and double stained profile of the cell

permeabilised and viable. It was optimisation of this latter subset which was required to ensure a good transfection and recovery rate of the cells.

2.12.2 CAT Assay

Once the conditions had been optimised Jurkat cells were transfected with constitutive CAT constructs (CMV-CAT plasmids, Invitrogen) to optimise the detection assay. Cells were electroporated, diluted in medium and cultured for 48 hours. Cell pellets were obtained after counting and centrifugation and were resuspended in ice cold 0.25M Tris-HCl pH 7.5 at $5x10^7$/ml. The cells were lysed by three freeze thaw cycles in liquid nitrogen and the supernatant was recovered by high speed centrifugation. The cytoplasmic extracts were frozen rapidly in liquid nitrogen and stored at -20°C until required.

Several methods have been described for the detection of CAT activity but a technique based on liquid scintillation counting (Neumann et al., 1987) was selected. An assay cocktail consisting of 100mM Tris-HCl pH 7.8, 1mM Chloramphenicol and 0.1mM ^{14}C acetyl Co A (ICN Cat. No. 13070L) was prepared. 200µl of this mix were then added to 50µl of cytoplasmic extract and overlaid with 5mls of scintillation fluid. Following the enzymatic transfer of the ^{14}C- acetyl group to the chloramphenicol, this molecule then partions into and activates the water immiscible scintillation fluid to cause an increase in detectable cpm. Any residual ^{14}C acetyl Co A remains in the layer below the scintillant. The samples were assayed at hourly intervals by β scintillation counting to enable the construction of graphs of extract volume and electroporation conditions against time .

2.12.3 Luciferase Assay

The cells were electroporated as described above and cultured for 24 hours. Pelleted cells were washed twice in PBS and then resuspended in Reporter Lysis Buffer (Promega Cat. No. E4030) at $1x10^7$/ml. After a 15 minute incubation at room temperature the cells were transferred to an eppendorf, cooled on ice and then vortexed for 10-15 seconds. Supernatants were recovered from a 15 second high speed centrifugation, underwent a single freeze-thaw and were stored at -70°C until required.

Luciferase activity was assessed by mixing 20µl of the extract with 100µl of luciferase assay reagent and measuring the light emitted using a Bio-orbit 1251 luminometer. If the machine is calibrated absolute values can be calculated otherwise results are expressed as relative activity compared to controls.

RESULTS

GENERAL AIMS OF THE WORK

The primary aims of this study were to investigate the role of CD28 as a costimulatory molecule in T cell activation, with particular reference to its regulation of IL-2 production. Previous studies have reported CD28-mediated effects on the gene promoter region which regulates IL-2 transcription although little is actually known concerning the participating role of CD28 signals as compared to those of the TCR. It was therefore proposed to further investigate the effects of CD28 on the IL-2 promoter and the signalling pathways utilised in the transduction of the signals from the cell surface to the nucleus. This was achieved using a variety of different techniques designed to provide qualitative and functional data arising from activation of the CD28 signalling pathway.

T CELL MODELS

Much of the previous work which has been carried out to establish the role of CD28 in transcriptional activation has been undertaken in immortalised cell lines such as Jurkat cells. Whilst these cells provide an easily available model system for such studies, their transformed phenotype means that responses observed may not always be an exact representation of the responses of resting T cells. Extrapolations from such *in vitro* to *in vivo* systems and responses should therefore be made with caution. The present study involved the development of a more physiological model system in which to investigate the role of CD28. Whilst Jurkat cells were still employed as controls in the establishment of the various techniques, more physiological T cell models were used where possible to ensure the relevance of the results. Comparison of the different cell models enabled the limitations of each model to be highlighted to aid their appropriate usage in future studies.

DATA

Variations in responses of different T cell blast and resting T cell cultures were commonly observed due to the varying sensitivity of the blood donors to different stimuli although the trends remained reproducible. It was therefore not possible to display the mean results of the repetitions despite a minimum of an n of 3 for each experiment. Data have therefore been shown as a representative experiment with the standard error representing variations in the triplicate readings. The absence of an error bar indicates an error of less than 5%.

CHAPTER 3 - THE ROLE OF CD28 AS A COSTIMULATORY MOLECULE

3.0 COSTIMULATION

Since the aims of the study centred around the role of CD28, it was important to first have an understanding of the effects on the T cells of CD28 stimulation alone and as a costimulatory molecule in the presence of TCR engagement. The antigenic signal can be mimicked by the use of cross-linked monoclonal antibodies to CD3 (review Janeway, 1992; Kuiper et al., 1994a; Costello et al., 1993) and many previous studies have utilised anti CD28 monoclonal antibodies to ligate the costimulatory surface receptor and induce activation (Kuiper et al., 1994a; Costello et al., 1993). However, since in the laboratory established B7-1 transfected CHO cell lines were available, initial experiments were undertaken to investigate the ability of these transfectants to costimulate anti-CD3 - stimulated T cells in proliferation assays. Figure 3.0 shows a representative assay comparing proliferative responses observed in resting purified peripheral blood T cells (A) and in peripheral blood mononuclear cells (PBMCs) (B). The cells were incubated with the appropriate stimuli for 72 hours before the assessment of proliferation by ^{3}H-Thymidine incorporation. As shown, whilst both cell types had negligible basal proliferation, PBMCs could be induced to proliferate in the presence of anti-CD3 alone whilst the purified T cells required both TCR and CD28 ligation. Addition of fixed CHO cells did not alter the responses but, in the presence of B7, proliferation increased in both cell types. This indicated that the B7 transfected CHO cells could provide adequate costimulation and that the induced response was specifically due to B7 and not to other features of the CHO cells.

Thus the observations made from these experiments indicated that the CHO-B7 transfectants were acting as an efficient costimulatory signal in these cells and that whilst the presence of B7 enhanced PBMC responses it was an absolute requirement for purified T cell activation.

3.1 USE OF PERIPHERAL BLOOD T CELL BLASTS

Due to the sensitivity limitations of several of the techniques used in this study, large numbers of T cells were required to enable detection and interpretation of the response. Whilst purified T cells could be regarded as the most physiological *in vitro* model of a resting T cell it was not possible to obtain a sufficient quantity to carry out all the required assays. PBMCs, although available in larger numbers, contain a mixed population of cells including monocytes and B cells and were therefore unsuitable for use as a specific T cell model. The preparation of T cell blasts from PBMC cultures, however, enabled expansion of the T cell population alone and resulted in a non-clonal

but responsive cell culture. Since these cultures were derived from resting cells it was hoped that they would prove to be a more relevant T cell model than the transformed Jurkat cell line. Thus PBMCs purified from human blood donations were stimulated with PMA and ionomycin to activate the T cells and the T cell blasts were allowed to grow out in culture for 4-10 days before being used for the various assays.

Proliferative responses were obtained for both the PMA/ ionomycin T cell blasts (figure 3.1A) taken from culture 8 days after the initial stimulation and for the Jurkat cells (B) as a comparison . Although compared to the unactivated cells basal proliferation of the T cell blasts was quite high, like the resting cells both anti-CD3 and B7 transfectants were required to induce proliferation although αCD3 alone did induce low levels of IL-2 . However, contrasting results were obtained with the Jurkat cells. These cells proliferate continuously in the absence of any extracellular stimuli and in an IL-2 independent manner. Consequently the basal proliferation of the Jurkat cells was extremely high making it difficult to assess the effects of a stimulus. As shown, stimulation of the Jurkat cells with anti CD3 in the presence or absence of B7 had little significant effect on basal proliferation levels nor did these stimuli induce any significant IL-2 production. Thus even from these initial experiments it appeared that the T cell blasts more closely represented resting T cells than the Jurkat cells.

3.2 EFFECTS OF PMA

Many of the previous studies undertaken to investigate the costimulatory role of CD28 have occurred in the presence of PMA and ionomycin (June et al., 1989). These two stimuli supposedly substitute for TCR ligation by directly activating PKC and triggering calcium entry respectively; downstream events which normally occur following engagement of the T cell receptor. As a comparison to the responses obtained following ligation of the TCR-CD3 complex using cross-linked antibody, the effects of PMA and ionomycin were also investigated in the different cell models.

Stimulation of resting T cells and T cell blasts with PMA and ionomycin induced proliferation and IL-2 output in both cell types (figure 3.2). This response was somewhat surprising especially in the resting T cells since stimulation of the TCR alone in the absence of a costimulatory signal is known to be insufficient for full activation (Harding et al., 1992). Furthermore, unlike the results seen with anti-CD3 antibodies, addition of B7 transfectants to the resting T cells appeared to decrease the responses whilst a slight increase in IL-2 production was observed in the T cell blast cultures. Thus PMA and ionomycin appeared to be providing different signals than the anti-CD3 antibody.

Interestingly, the observed responses of Jurkat cells revealed that whilst they were relatively unresponsive to stimulation via their surface receptors they were highly sensitive to stimulation with PMA and ionomycin. Incubation of the Jurkat cells with PMA alone or in the presence of ionomycin dramatically inhibited the basal proliferation whilst inducing large amounts of IL-2 (figure 3.3). Addition of B7 transfectants to the PMA/ ionomycin stimulated Jurkat cells had little effect on the PMA-induced inhibition of proliferation but induced a further increase in IL-2 production. Since PMA and ionomycin are proposed to be activating stimuli this negative effect on proliferation was unexpected and implied a substantial difference in signalling outcome in the transformed Jurkat cells compared to normal T cells.

Following this observation further studies were undertaken to investigate the effects of PMA on Jurkat cells. Assessment of cell viability as measured by trypan blue exclusion was inconclusive since although there appeared to be fewer cells in the stimulated cultures at the end of the experiment, those cells that remained were still viable. The PI stained cytospins were also difficult to interpret due to low level staining and the small number of cells examined. FACS analysis of a timecourse of PMA stimulation provided the most information and showed an increase in Jurkat cell death over time, a loss of S phase cells and an accumulation of the remaining live cells in G2 phase (figure 3.4) indicating the cells were undergoing cycle arrest prior to death.

3.3 COSTIMULATION OF A SUBOPTIMAL PMA INDUCED RESPONSE

Since the PMA and ionomycin stimulation appeared to be providing a maximal response it was possible that the high concentration of PMA (30ng/ml) may have been activating both the TCR and costimulatory pathways. It was therefore decided to reduce the concentration of PMA used in an attempt to separate activation of the two signalling pathways and observe a costimulatory role for B7 in mitogenically-activated normal T cells. PMA titration proliferation profiles were obtained for Jurkat cells, resting T cells and the T cell blasts (figure 3.5) which revealed several interesting points. The three cell types demonstrated different sensitivities to PMA stimulation both in respect to each other and when comparing effects on proliferation and IL-2 production. Interestingly in all three cell types induction of IL-2 decreased at the highest dose of PMA implying a toxic effect.

As observed during the initial assays the addition of PMA to Jurkat cells inhibited their proliferation whilst stimulating IL-2 production. However although the effect on proliferation was maximal at 0.03ng/ml, this did not induce IL-2 production which peaked at 3ng/ml PMA. This indicates different PMA sensitivities of the two activation events and implies at least two different targets for PMA. Interestingly in the presence of

high doses of PMA the inhibitory effect on proliferation was partially reversed which may indicate the activation of an additional pathway which antagonises the inhibition of proliferation.

Resting T cells, in the presence of ionomycin, required a minimum PMA concentration of 0.03ng/ml PMA to observe proliferation whilst a minimun threshold of 0.3ng/ml was required for IL-2 output. Therefore as observed with the Jurkat cells IL-2 production appears to require a higher level of stimulation than proliferation. However, in the resting cells both proliferation and IL-2 production reached a peak at 0.3ng/ml indicating that with respect to IL-2 production these cells had an increased sensitivity to PMA compared to the Jurkat cells.

The T cell blasts responded almost as a mirror image of the Jurkat cells. The minimum dose of 0.003ng/ml induced a high level of proliferation which was maintained over the titration. The T cell blasts were also the most responsive to PMA following the assessment of IL-2 production implying that the activated cells had a lower PMA threshold requirement for IL-2 production than either the transformed or resting T cells.

Having established the responses of different cell types to suboptimal PMA concentrations the effects of B7 alone ([PMA]=0) and as a costimulus to the PMA/ ionomycin responses, were investigated. Figure 3.6A shows the effect of B7 on suboptimal PMA- stimulated Jurkat cells. The addition of B7 transfectants alone had no effect on Jurkats however, in the presence of PMA concentrations above 0.03ng/ml the B7 stimulation did restore some of the proliferative ability of the cells and considerably increased their IL-2 ouput. As these effecs were only observed at high doses of PMA (>0.03ng/ml) this indicated a requirement for a certain threshold level of PMA-induced activation of the cells.

A similar PMA/ionomycin/ B7 synergy was observed in resting T cells (figure 3.6B) but only in the presence of very low concentrations of PMA (< 0.3 ng/ml). As shown, 0.003ng/ml PMA in the presence of ionomycin did not induce proliferation alone but did so in the presence of the B7 transfectants. This indicates that the presence of B7 increases the sensitivity of these cells to PMA stimulation and implies a synergy between the two signals. This stimulation, however, did not result in IL-2 production which required a minimum PMA concentration between 0.003ng/ml and 0.03ng/ml in the presence of B7. The lower doses of PMA required for activation in resting T cells indicated that these were more representative of physiological concentrations and that the higher doses used routinely *in vitro* may invoke unphysiological responses due to cytotoxicity.

In the case of the PMA/ ionomycin blasts, B7 stimulation enhanced the responses seen with PMA and ionomycin alone at all concentrations of PMA but most significantly at 0.3ng/ml (figure 3.6C).

Following this study, for all subsequent experiments involving PMA stimulation, a final concentration of 0.3ng/ml was used for the Jurkat cells and T cell blasts and 0.03ng/ml for the resting T cells which was deemed to be sufficient to give a submaximal response in each of the cell types so allowing the effects of additional stimulations to be observed.

3.4 FACS ANALYSIS OF SURFACE EXPRESSION

Prior to their use in experimental assays the T cells routinely underwent FACS analysis to check the viability, purity and surface molecule expression levels. Cells were stained for a number of different surface markers chosen to indicate the identity and activation state of the cell and then analysed by FACS. Figure 3.7 shows the diagramatic FACS profile of Day 8 T cell blasts whilst those for Jurkats and resting T cells are shown in figures 3.8 and 3.9 respectively. The percentage of positive cells and MFI for each surface molecule are also shown in Tables I to III. Although the expression profiles for the cell types were similar some differences were evident. Jurkats and resting T cells were IL-2Rα negative ($CD25^-$) explaining their lack of response to exogenous IL-2 whereas the T cell blasts displayed varying degrees of positive staining. Similar observations were made for the MHC class II molecule which was expressed by the activated T cell blasts but not by the Jurkat cells or resting T cells. All cells were CD2, CD3 and CD28 positive to varying degrees and B7 negative.The majority of resting T cells and blasts were $CD4^+$ although a small population were found to be $CD8^+$ T cells.

Figure 3.10 shows the surface expression of the transfectants used. Again these were routinely assayed to ensure stable expression levels of the relevant surface markers. CHO cells were routinely negative for all relevant surface molecules and were therefore used as negative controls in experiments requiring transfectant stimulation.

3.5 SUMMARY

Initial experiments indicated that resting T cells could be induced to proliferate and produce IL-2 using a combination of cross-linked anti-CD3 antibody and fixed CHO-B7 transfectants. High concentrations of PMA in combination with ionomycin provided all the necessary activation signals whilst suboptimal PMA doses could be effectively costimulated by B7. The T cell blasts also required two signals for full activation but were fully activated by extremely low doses of PMA in the presence of ionomycin. However, the T cell blasts appeared to be a more physiological model of a resting cell

than Jurkat cells which were unresponsive to surface stimulation and inhibited by PMA. Further studies will be required to decipher the exact effects of PMA in T cells as high doses appear to have additional effects to activation of the TCR pathway. A comparison of the responses observed in the presence and absence of costimulation using anti-CD3 or low dose PMA/ ionomycin stimulated cells should enable some of the effects of CD28-mediated signals to be identified.

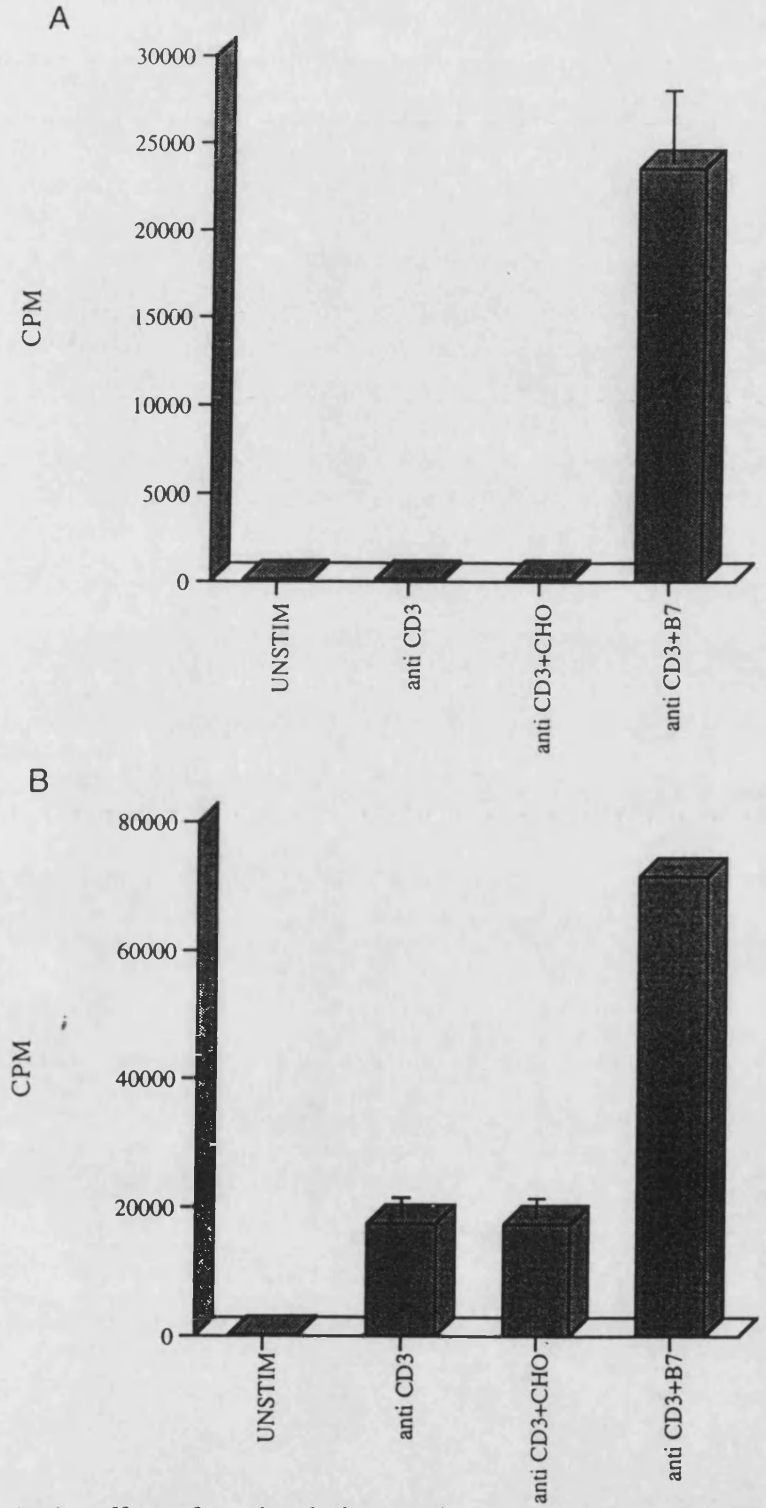


Figure 3.0 The effect of costimulation on the proliferative responses of αCD3 stimulated Resting T cells and PBMCs

Resting T cells (A) and PBMCs (B) were left unstimulated or were incubated with αCD3 (1μg/ml) alone or in the presence of fixed CHO cells or fixed B7 cells for 72 hours. Proliferation was measured by ^{3}H-Thymidine incorporation. Data are the triplicate mean of a single representative experiment.

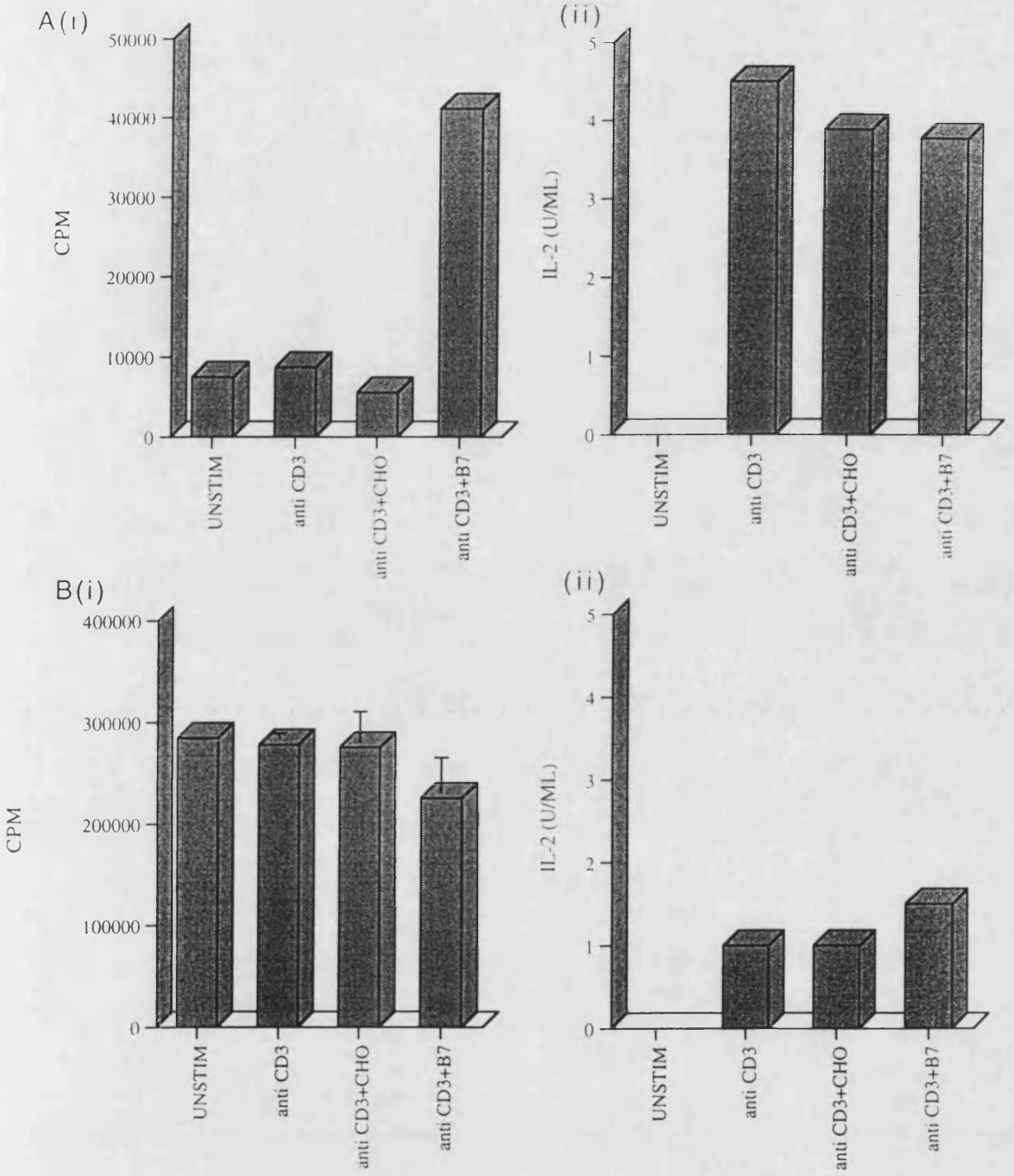


Figure 3.1 The effect of costimulation on the proliferative responses and IL-2 production of αCD3 stimulated PMA/ ionomycin T cell blasts and Jurkat cells

PMA/ionomycin T cell blasts (A) and Jurkat cells (B) were left unstimulated or were incubated with αCD3 (1μg/ml) alone or in the presence of fixed CHO cells or fixed B7 cells for 72 hours. Proliferation (i) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (ii) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

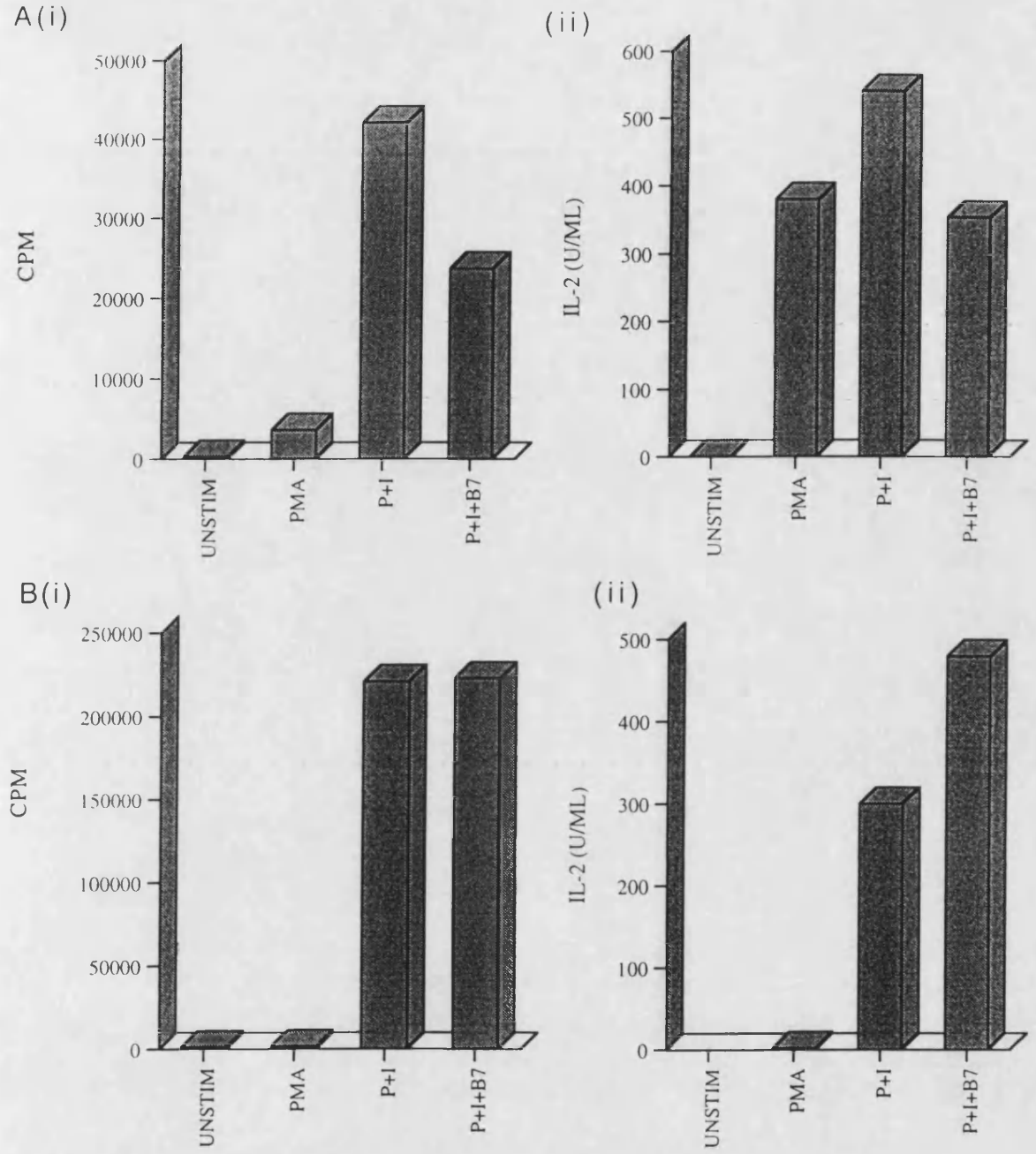


Figure 3.2 The effect of costimulation on the proliferative responses and IL-2 production of PMA/ ionomycin stimulated Resting T cells and T cell blasts

Resting T cells (A) and PMA/ionomycin T cell blasts (B) were left unstimulated or were incubated with PMA (30ng/ml) and ionomycin (1μM) alone or in the presence of fixed CHO cells or fixed B7 cells for 72 hours. Proliferation (i) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (ii) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

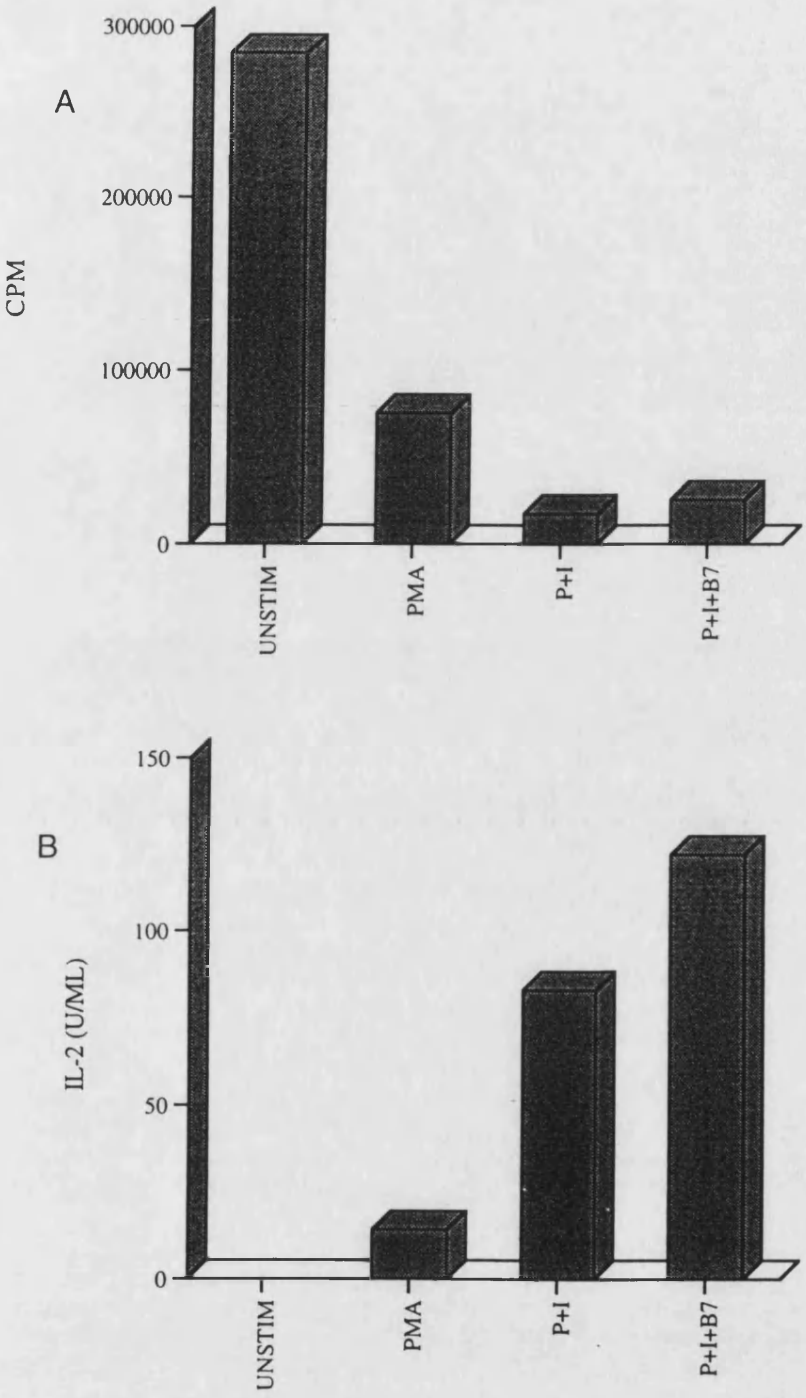
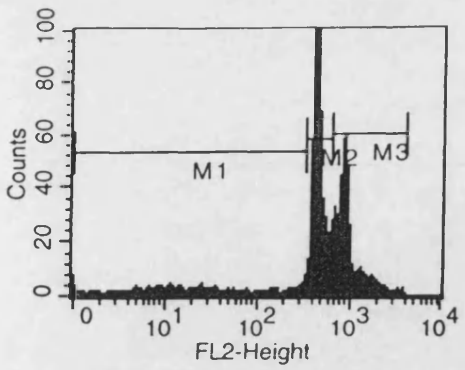


Figure 3.3 The effect of costimulation on the proliferative responses and IL-2 production of PMA/ ionomycin stimulated Jurkat cells

Jurkat cells were left unstimulated or were incubated with PMA (30ng/ml) and ionomycin (1μM) alone or in the presence of fixed CHO cells or fixed B7 cells for 72 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

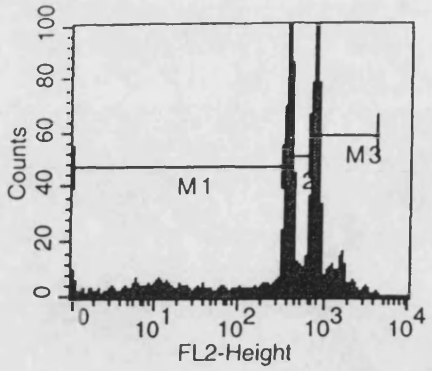
i



JURKAT (OX) UNSTIM 24HR

Marker	% Total	Mean
All	100.00	579.67
M1	8.28	94.91
M2	60.80	446.65
M3	31.68	962.28

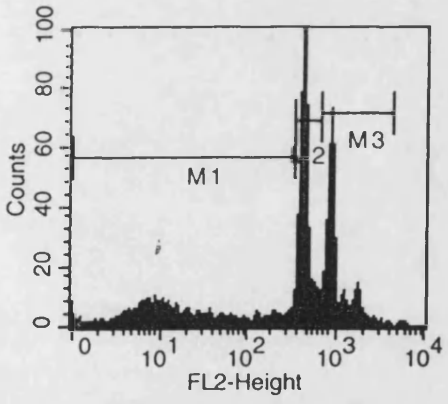
ii



JURKAT (OX) PI 24HR

Marker	% Total	Mean
All	100.00	539.03
M1	14.06	90.55
M2	40.70	425.17
M3	38.94	869.58

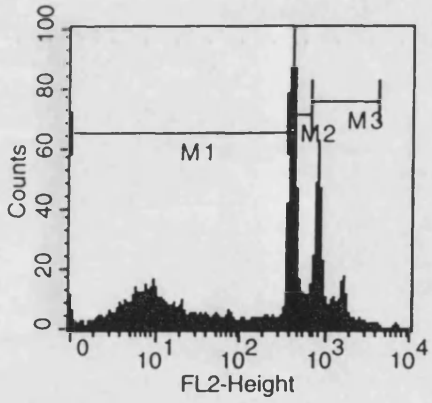
iii



JURKAT (OX) PI 48

Marker	% Total	Mean
All	100.00	499.76
M1	25.86	67.90
M2	44.86	420.40
M3	29.10	994.15

iv



JURKAT (OX) 72 HR PI

Marker	% Total	Mean
All	100.00	421.50
M1	38.86	49.72
M2	36.26	410.57
M3	25.72	994.24

Figure 3.4 The effect of PMA on Jurkat cells

Jurkat cells were left unstimulated (i) or were incubated with PMA (30ng/ml) for 24 hours (ii), 48 hours (iii) or 72 hours (iv). The cells were then washed and stained with propridium iodide before FACS analysis. Data is plotted on a log scale to show sub G_0 (M1), G_1 / S (M2) and G_2 (M3).

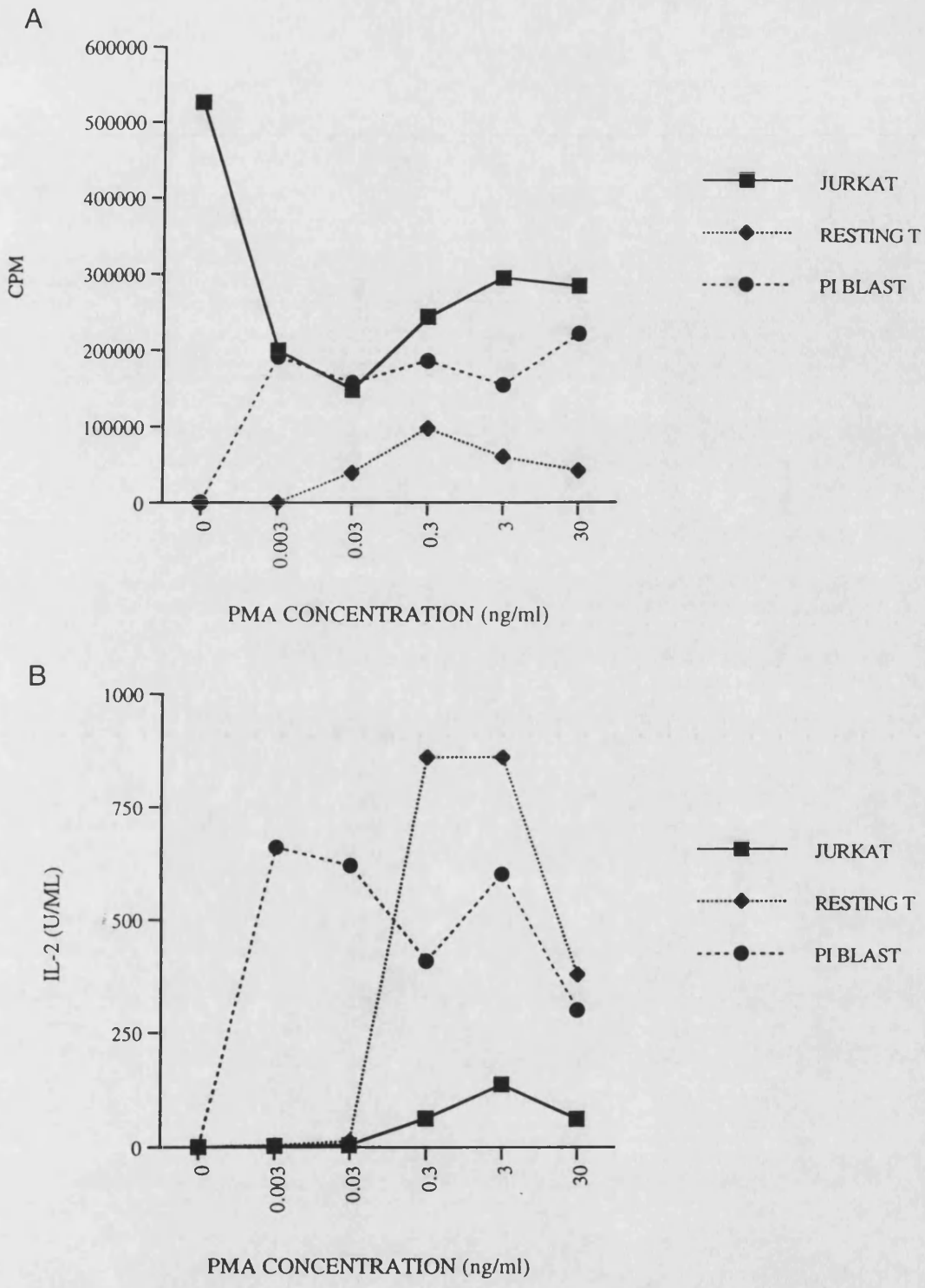


Figure 3.5 The effect of PMA concentration on the proliferative responses and IL-2 production of different cell types

Cells were left unstimulated or were incubated with increasing concentrations of PMA in the presence of ionomycin (1μM) for 72 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

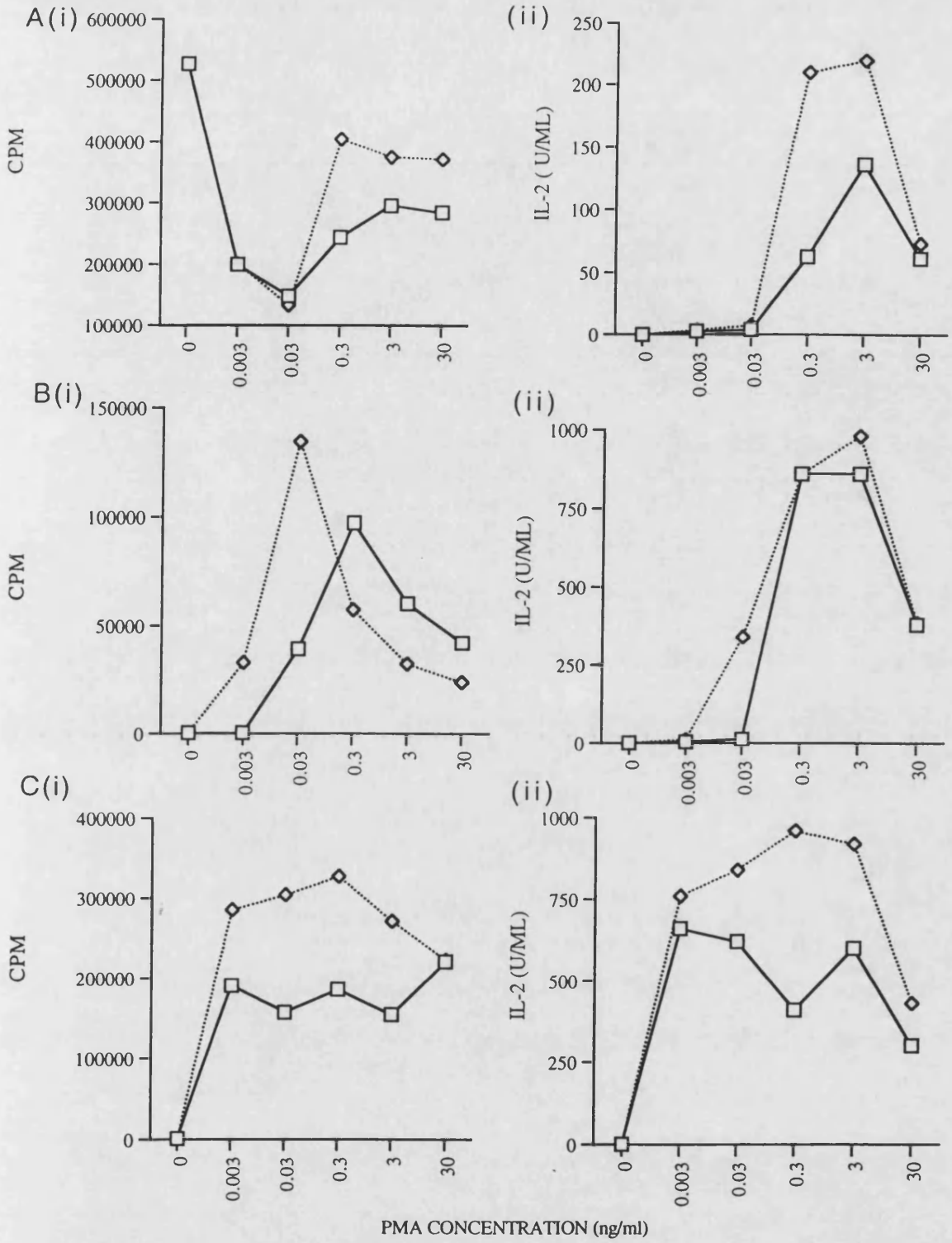


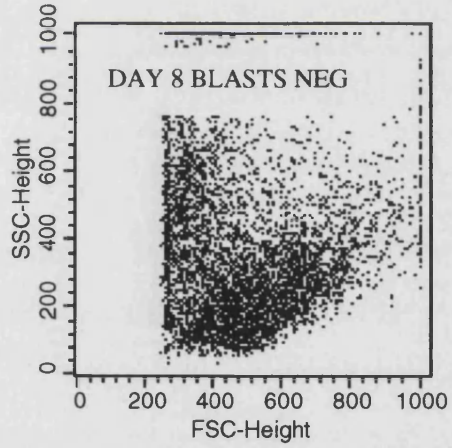
Figure 3.6 The effect of costimulation on the proliferative responses and IL-2 production of different cell types stimulated with suboptimal concentrations of PMA

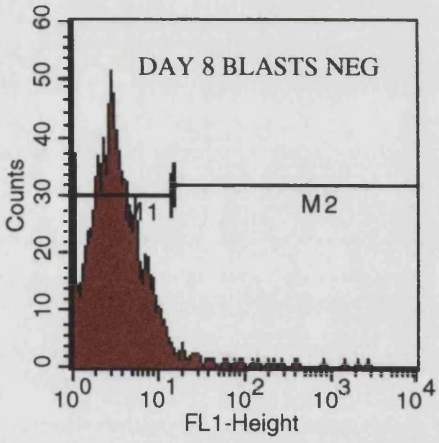
Jurkat cells (A), Resting T cells (B) and T cell blasts (C) were left unstimulated or were incubated with ionomycin (1μM) and increasing concentrations of PMA alone (□) or in the presence of fixed CHO-B7 transfectants (◇) for 72 hours. Proliferation (i) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (ii) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

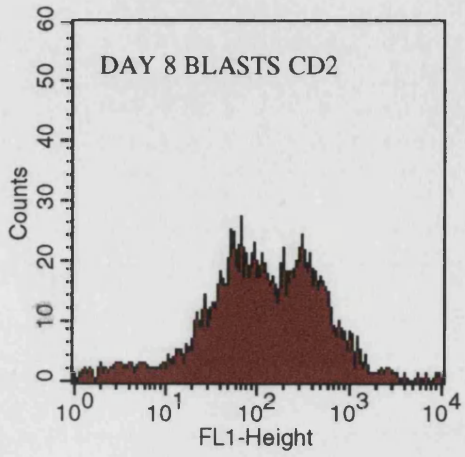
Table I- FACS analysis of the surface expression of day 8 T cell blasts

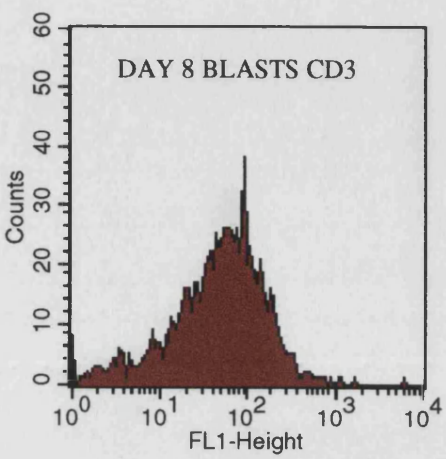
Cells were fluorescent stained for a number of surface T cell molecules and analysed by FACS. Results are shown as percentage of cells with the mean fluorescent index (MFI).

SURFACE MOLECULE	MARKER	% OF TOTAL	MFI
NEG	M1	98.6	3.7
CD2	M2	95.3	218
CD3	M2	89.1	83.6
CD4	M2	54.1	37.7
CD8	M1	78.5	4.6
CD25	M1	69.1	5.5
CD28	M2	73.6	60
CD80	M1	97.5	3.6
HLA-DR	M1	64.7	5.6









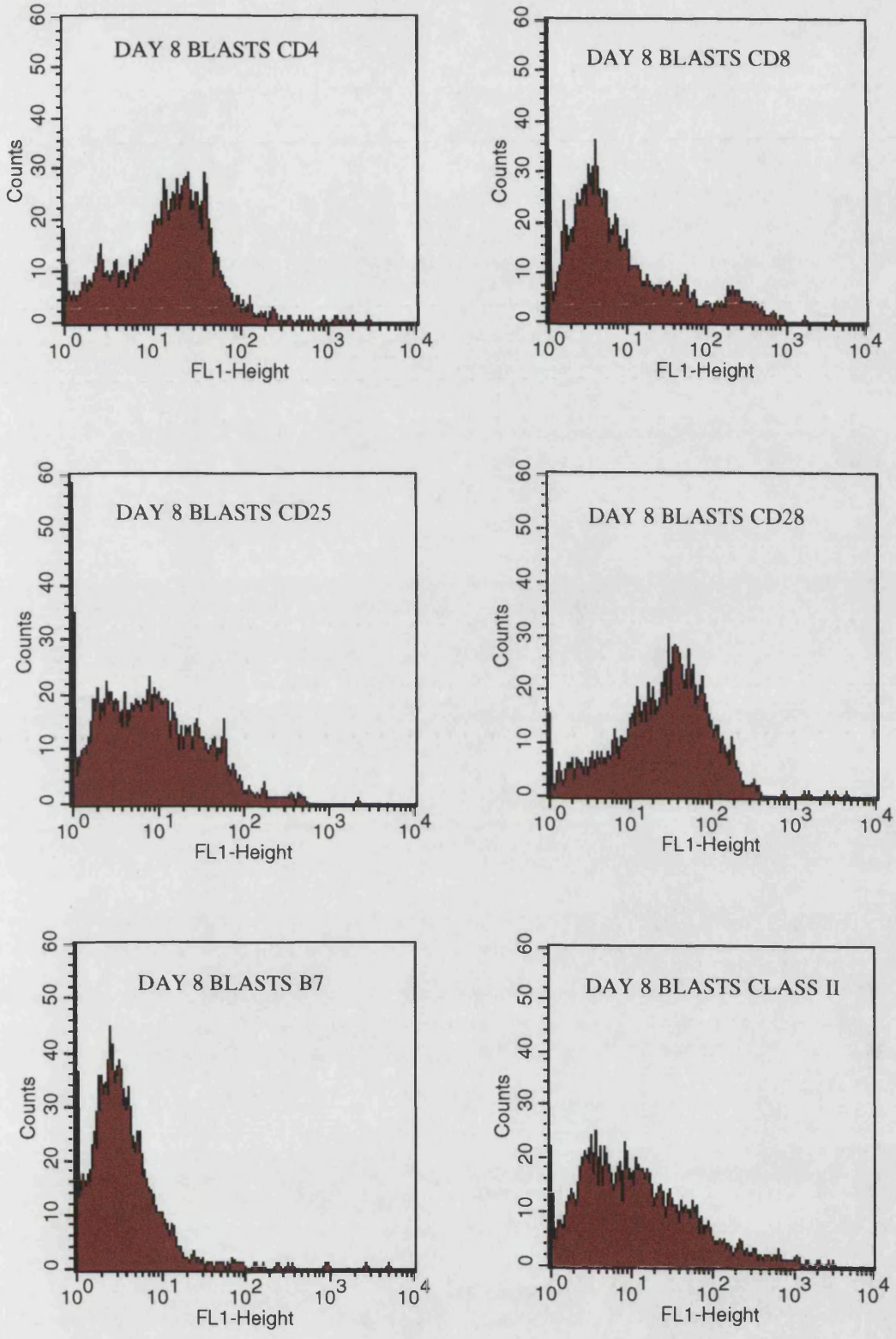
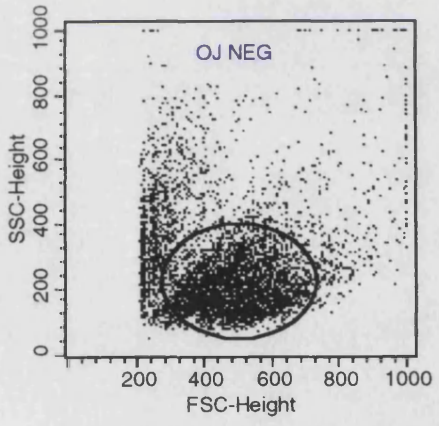
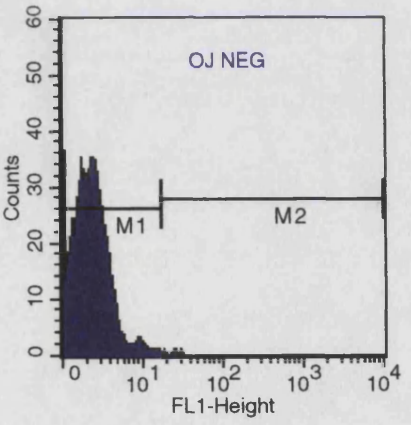


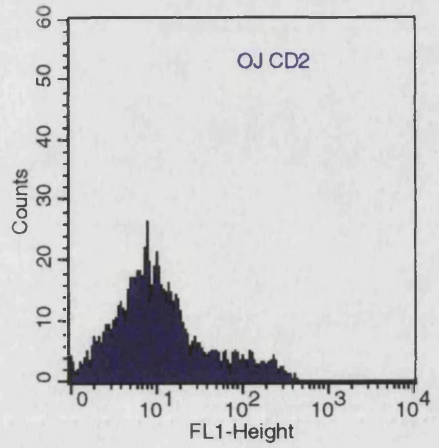
Figure 3.7 FACS analysis of Day 8 T cell blasts

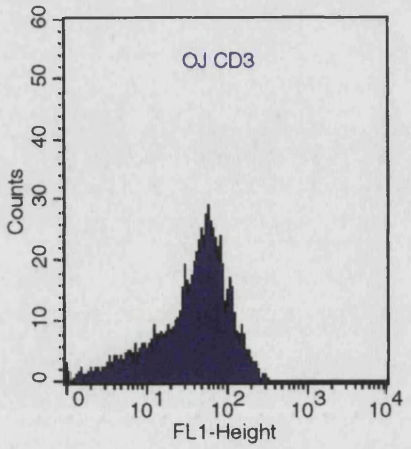
Cells were fluorescent stained for a number of surface T cell molecules and analysed by FACS. Results are shown as log histograms of surface staining.

SURFACE MOLECULE	MARKER	% OF TOTAL	MFI
NEG	M1	95	2.5
CD2	M1	79	7.5
CD3	M2	83	61.4
CD25	M1	97	2.6
CD28	M2	94	79
CD80	M1	100	2.4
HLA-DR	M1	100	2.4









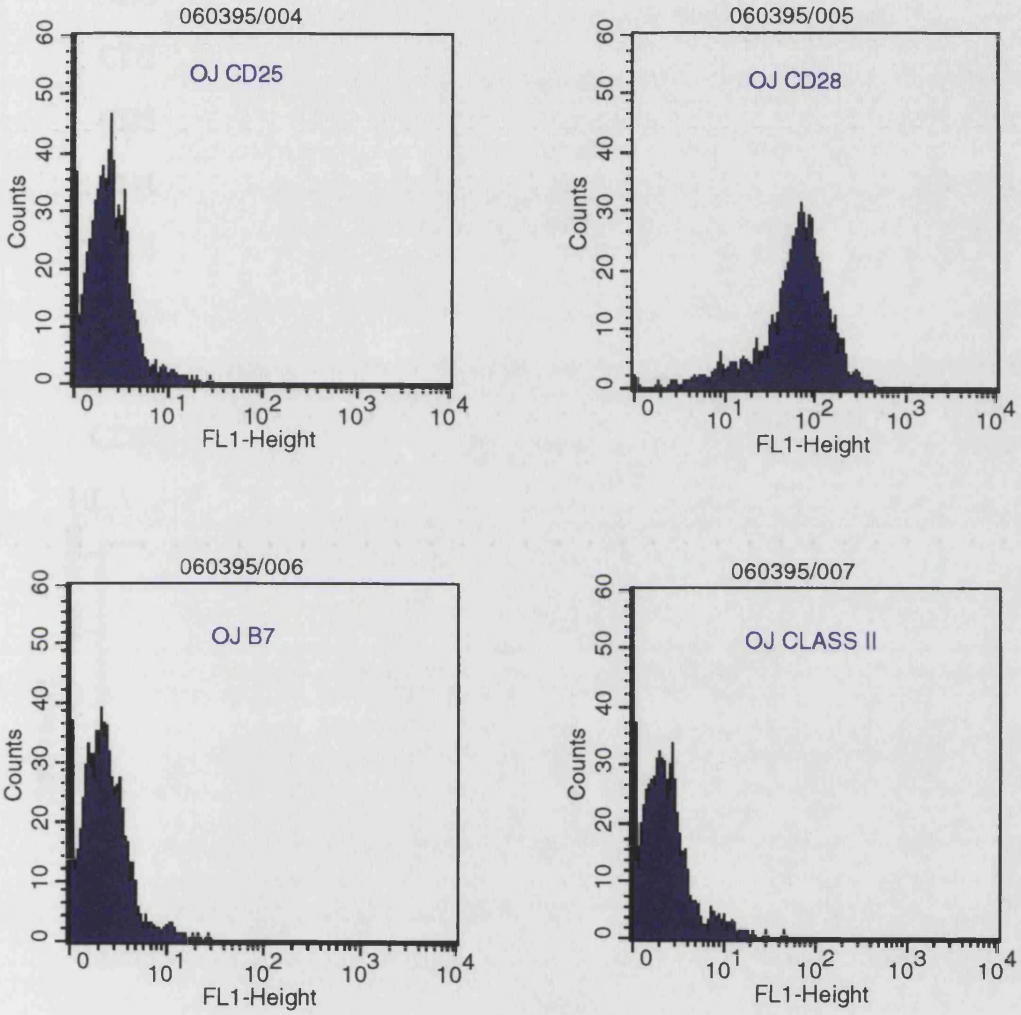


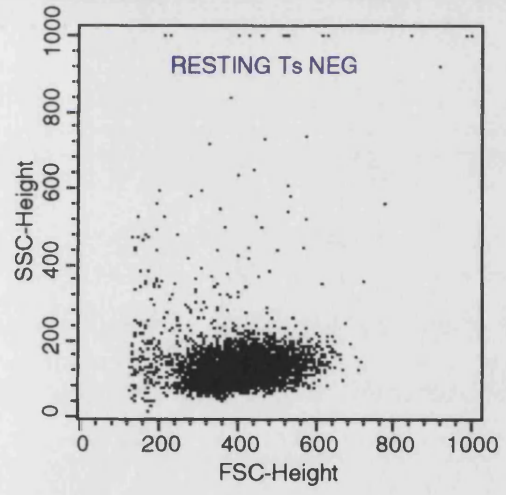
Figure 1.8 FACS analysis of the surface expression of Jurkat cells

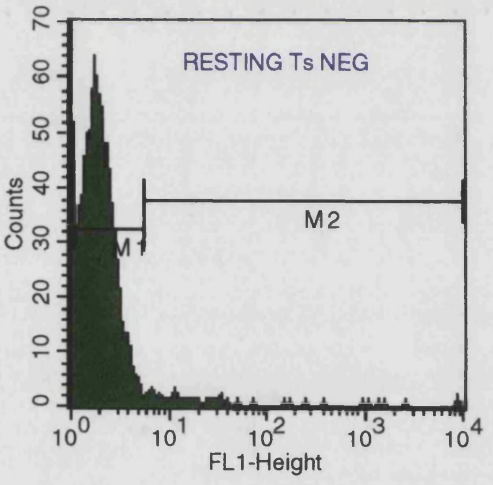
Cells were fluorescent stained for a number of surface T cell molecules and analysed by FACS. Results are shown as log histograms of surface staining.

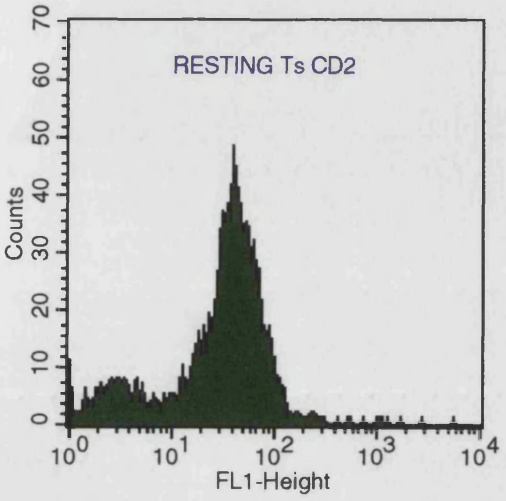
Table III FACS analysis of the surface expression of Resting T cells

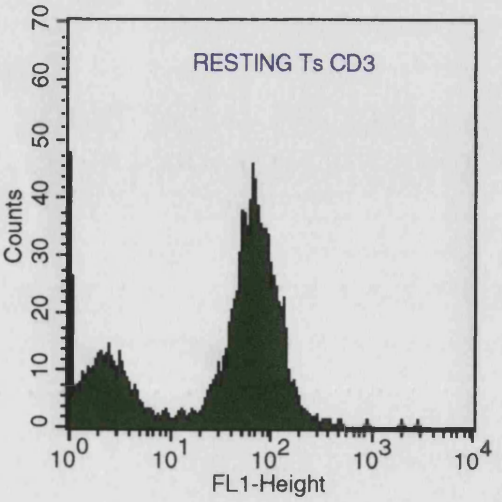
Cells were fluorescent stained for a number of surface T cell molecules and analysed by FACS. The results are shown as of percentage of cells with the MFI.

SURFACE MOLECULE	MARKER	% OF TOTAL	MFI
NEG	M1	98.7	2.1
CD2	M2	88.2	47.4
CD3	M2	76.7	74.1
CD4	M2	59.6	31.8
CD8	M1	77.1	2.5
CD25	M1	98.2	2.2
CD28	M2	69.8	19.8
CD80	M1	98.4	2.4
HLA-DR	M1	95.4	2.4









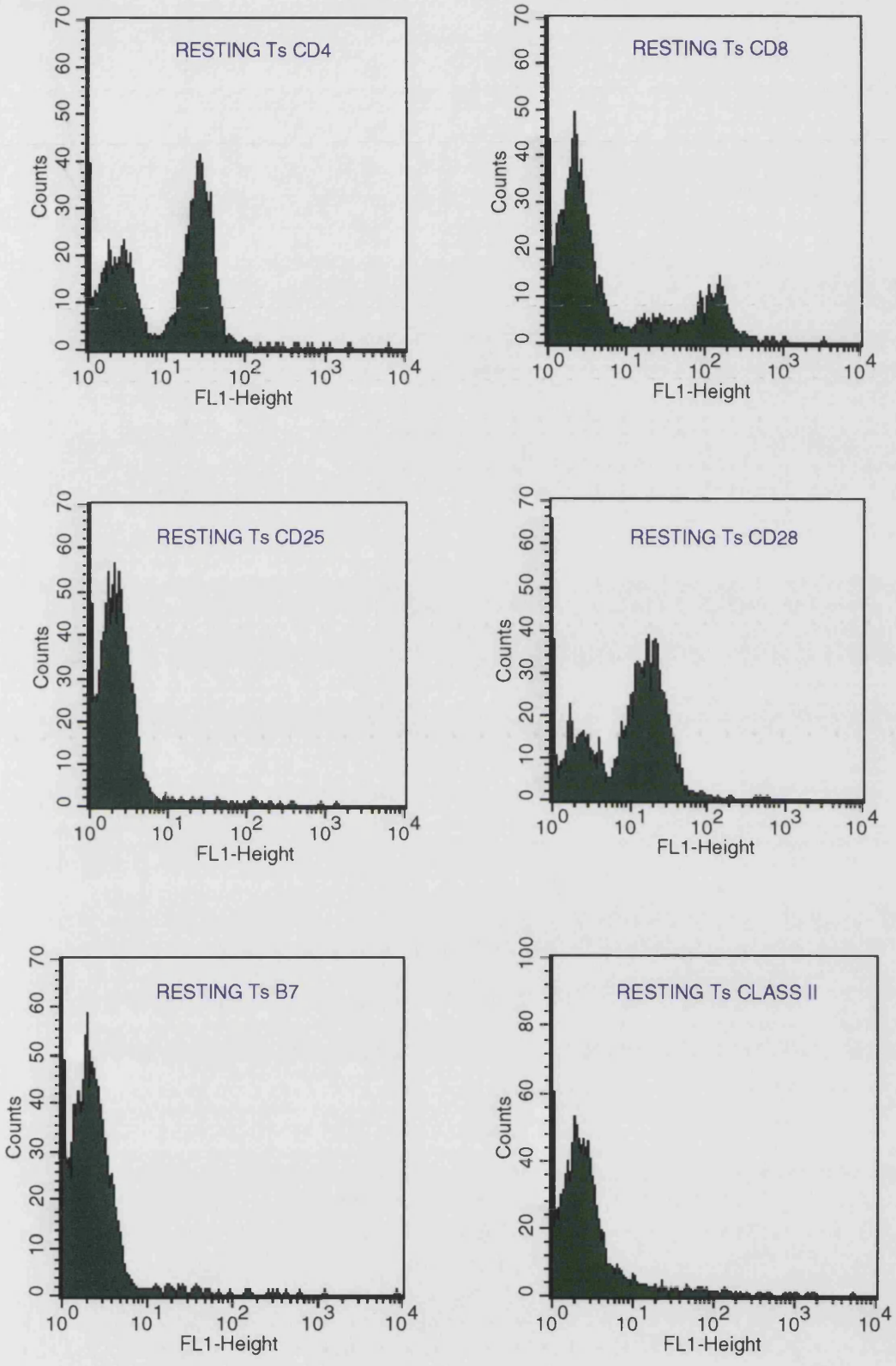


Figure 3.9 FACS analysis of Resting T cells

Cells were fluorescent stained for a number of surface T cell molecules and analysed by FACS. Results are shown as log histograms of surface staining.

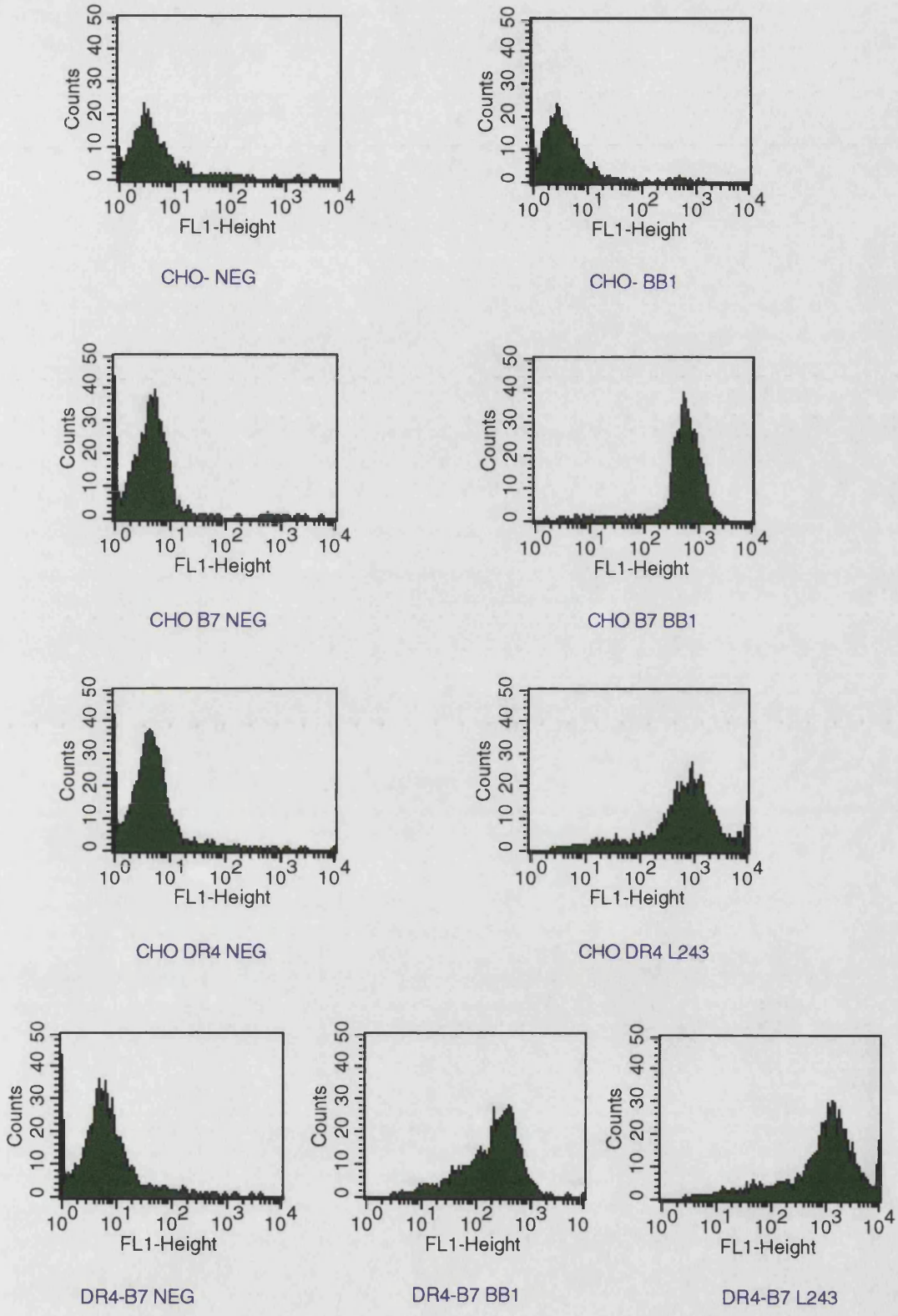


Figure 3.10 FACS analysis of the surface expression of transfected CHO cells

CHO transfectants were stained with appropriate antibodies or media to detect surface expression of the transfected molecules. CHO cells were also stained as negative controls. Data are shown as log histograms indicating the extent of surface staining.

3.6 DISCUSSION

The data obtained from my studies highlighted the requirement of normal T cells to receive two signals to induce full activation whilst PBMCs were activated by αCD3 alone. This reflected the presence of costimulatory molecules on professional APCs present in the PBMC population which were removed during the purification of the T cells. Furthermore the failure of αCD3 alone to activate the resting cells validated the purification process. Since the PBMC response can be blocked by the CTLA-4Ig chimeric molecule (Tan et al., 1993) this would suggest that B7 is involved in the activation of these cells. In agreement with previous reports (Linsley et al., 1991a; Koulova et al., 1991; Gimmi et al., 1991) the requirement for B7 to enable the activation of purified T cells by αCD3 was confirmed by the addition of fixed CHO-B7 transfectants to a purified T cell population stimulated with anti-CD3 antibodies. As antibody alone induced no observable change whilst the combined stimulation resulted in proliferation and IL-2 production, this therefore established that B7 was providing the critical second signal and that the transfectants could adequately mimic the natural CD28 ligand. Furthermore these experiments proved that glutaraldehyde fixation of the transfectant cells, whilst inhibiting their proliferation, leaves the surface molecules intact and functional. Previously many groups have used anti-CD28 monoclonal antibodies to activate the costimulatory pathway instead of the B7 ligand, however, this has often resulted in misleading observations as different signalling events can be observed depending upon whether the antibody is soluble or cross-linked (Ledbetter et al., 1990). Furthermore multivalent antibody ligation of CD28 has been shown to induce a calcium flux (Linsley and Ledbetter, 1993) and yet this has not been observed using the natural ligand, B7.

All further experiments undertaken in the study therefore involved the use of fixed CHO-B7 transfectants as a source of costimulation as it was hoped that the results observed would also be more physiologically relevant than those obtained using anti-CD28 antibodies. In fact preliminary experiments in the laboratory indicated the transfectants provided a more effective costimulatory signal as observed by the degree of proliferation they induced compared to antibody stimulated cells (D.Sansom, A.Wilson, personal communication).

Immortal T cell lines such as Jurkat cells and Hut 78 cells, derived from leukaemic populations and virally transformed, have long been established as laboratory models of T cell function. However, whilst they retain some phenotypic features of normal T cells, several of their intracellular pathways have become constitutively activated or altered. From the literature it appears that whilst these immortal cells are routinely used, current understanding concerning the regulation of their intracellular signalling pathways is

extremely limited. In the initial studies it was observed that Jurkat cells undergo maximal proliferation in culture in the absence of IL-2 and IL-2 receptor. Furthermore the Jurkat cells appeared to be fairly unresponsive to surface receptor stimulation in that αCD3 or B7 stimulation had very little effect on proliferation and did not induce IL-2 production. Thus whilst the Jurkat cells provided a useful control model for mitogenically activated signalling pathways an alternative T cell model was required. The second aim of this work was therefore to investigate more physiological models of T cell function to enable investigation of the role of the costimulatory signal in T cell activation and IL-2 production.

A study of T cell blast responses at various time points after activation revealed that these cells could easily be stimulated to proliferate, so rapidly increasing the total cell number. Furthermore, expansion of the PMA/ ionomycin stimulated population helped to decrease the differences in the responsiveness of the T cells obtained from different donors making inter-experiment comparisons more relevant. Thus large numbers of PMA/ ionomycin T cell blasts were prepared and cultured under standardised conditions for use in the various assays. Unlike the constitutively activated Jurkat cells, these T cell blasts could be quiesced before being used, so enhancing their similarity to a resting T cell yet unlike clonal populations of T cells the blast cultures retained the varied specificity of a purified T cell population. Stimulation of the quiesced T cell blasts with αCD3 and B7 revealed that like purified T cells they required two signals to induce proliferation indicating that the T cell blasts were not constitutively activated.

Further comparative experiments using Jurkat cells, T cell blasts and resting T cells were then carried out to assess the similarities and differences of the various models. Interestingly the addition of stimuli which usually promote proliferation in normal T cells, either had no effect or were inhibitory to the Jurkat cells. Preliminary data obtained from PMA-stimulated Jurkat cells in the laboratory indicated that the responses observed were substantially different from those seen in normal T cells. The results showed that whilst stimulation of resting T cells or T cell blasts with PMA, in the presence of ionomycin, induced proliferation, stimulation of Jurkat cells with PMA and ionomycin was inhibitory, yet IL-2 production occured in all three cell types. Thus it appeared that in Jurkat cells, there was an inverse correlation between proliferation and IL-2 production indicating that these signalling pathways were differentially regulated by phorbol esters. These data could be accounted for by the presence of different PKC isotypes which induce stimulatory or inhibitory signals following PMA activation.

The observation that stimulation of Jurkat cells with PMA inhibited their basal proliferation implied that this constitutively activated pathway was negatively regulated by PKC. Further investigation of this effect indicated that the cells were dying when

incubated with PMA for extended periods (>24 hrs). FACS analysis of these stimulated cells showed an inhibition of cell cycle and an increase in cell death compared to unstimulated populations of cells. The addition of PMA appeared to prevent the cells from entering mitosis with the subsequent accumulation of cells in G_2 phase. The loss of S phase cells appeared to confirm the halt in cell cycle progression induced by the PMA stimulus whilst the appearance of a population of the cells in the sub G_0/G_1 region indicated an increase in cell death compared to unstimulated cells cultured under the same conditions. It is possible that this increase in cell death is due to an induction of apoptosis although further studies would be required to confirm this. Since Jurkat cells proliferate constitutively in the absence of exogenous stimuli this may indicate they have a constitutively activated component of the calcium signalling pathway. Thus if PMA inhibits the PKC-regulated part of the signalling pathway the resultant calcium signal, in the absence of other signals, may be inducing apoptosis. This is just one possible interpretation of the data and these events will require further investigation to establish the exact effects of PMA in these cells.

The confusion associated with the observed differences in mitogen- induced responses between Jurkat and normal T cells was heightened by the limited understanding of the effects of phorbol esters such as PMA on intracellular signalling pathways and the variability in the dose used. A study of the literature indicated that the concentration of PMA used by different groups to induce T cell activation, varied considerably, usually between 1 and 50ng/ml. As the aim of this study was to investigate the effects of costimulation it was necessary to find a dose of PMA which could be costimulated by CD28 engagement.

PMA is commonly used as an activator of T cells in combination with another stimulus which triggers a calcium signal, frequently the calcium ionophore, ionomycin (Truneh et al., 1985) or the CD2 stimulator, PHA (Weiss and Stobo, 1984). Interestingly whilst activation of PKC by PMA, in the presence of calcium, is supposed to represent a triggering of the TCR pathway alone, PMA and ionomycin cause full activation of T cells whereas TCR engagement alone does not. In fact TCR stimulation in the absence of a costimulatory signal is often the cause of anergy (Harding et al., 1992). Thus it appears that PMA is not just stimulating the TCR-related PKC pathway but is also activating additional pathways which are normally regulated by the costimulatory molecule. In support of this hypothesis is the finding that in normal T cells low dose PMA (<1ng/ml) could be effectively costimulated by B7 whereas high dose PMA (30ng/ml) induced a maximal response and implies the existence of at least two PMA targets on different pathways which display different sensitivities to activation by PMA. In addition this may explain the "rebound" effect found in Jurkat cells where high dose PMA partially reversed the inhibition of proliferation observed at lower doses. In Jurkat cells it appears

that low dose PMA may activate the TCR pathway only and induce cell death whereas higher doses may also activate the costimulatory pathway which delivers a protective signal.

Attempts to identify target sites for PMA-induced effects have lead several groups to investigate the different signalling effects observed in PMA-treated cells. Two recent reports have indicated the involvement of PMA in both the TCR and CD28 signalling pathways. Williams et al (1995) reported an upstream role for PKC in ras signalling whilst Hutchcroft et al (1995) reported an inhibition of the association between CD28 and PI3 Kinase in the presence of PMA. However, if this observation is true then the protective effect of the CD28 costimulation in Jurkat cells must involve an alternative signalling pathway to PI3 Kinase. Recently Cifone et al (1995) have reported that acidic sphingomyelinase, which is also associated with CD28 signalling, can be activated by DAG, the physiological intracellular activator of PKC. Thus as PMA activates PKC by mimicking DAG it may also be capable of activating acidic sphingomyelinase. This is proposed to be upstream of the generation of ceramide which has been reported to activate the supposedly PMA-insensitive atypical PKC isoenzyme PKCζ (Muller et al., 1995b). However, there are numerous PKC isotypes of which very little is known so the participation of a second PMA-sensitive isotype in this signalling pathway is possible. Interestingly the protective effect of CD28 against apoptosis has been proposed to be mediated via an increase in bcl-x expression (Boise et al., 1995) whilst PMA stimulation has been shown to protect against some forms of glucocorticoid-induced apoptosis (Walker et al., 1993; Gomez et al., 1994) and since glucocorticoids target the transcription factors which have been reported to be partially regulated by CD28 (Auphan et al., 1995; Scheinman et al., 1995), the link between the CD28 costimulatory pathway, PMA and protection against apoptosis appears to be strengthening. Unfortunately such assays are frequently carried out in different cell types so it is difficult to extrapolate the findings across into normal cells. Furthermore if PMA is activating more than one signalling pathway, the overall response will be a summation of the interaction of these pathways and therefore less easy to define in terms of isolated events. Therefore until there is a greater understanding of the expression and PMA sensitivity of different PKC isotypes and a development of more specific isotype activators and inhibitors, data obtained in PMA stimulated cells should be interpreted with caution.

These initial assays have highlighted the requirement for two signals to fully activate normal T cells and that the second signal can be efficiently provided by the fixed CHO-B7 transfectants. Furthermore it has been shown that high doses of PMA in combination with ionomycin can induce full activation by stimulating both the TCR and costimulatory pathways but that low dose PMA can be effectively costimulated by B7. These

experiments also highlighted some of the problems associated with using transformed cell lines such as Jurkat cells as models of resting T cells due to their altered signalling mechanisms.

Resting T cells are the best *in vitro* model for the investigation of T cell function but due to the extremely low physiological intracellular concentrations of the proteins it is often difficult to detect responses without using very large numbers of cells which are difficult to obtain. The use of quiescent T cell blasts is thus more amenable and from these initial experiments it appears that these cells more closely resemble resting T cells than Jurkat cells do. The use of the T cell blasts to study the role of CD28 will hopefully provide more relevant information although comparative studies in Jurkats will increase the understanding and therefore the usefulness of this T cell model.

Therefore having established that the T cell blasts could be costimulated by B7 in the presence of suboptimal doses of PMA, I proposed to investigate the effects of this costimulation on transcription factor induction and IL-2 production.

CHAPTER 4 - INVESTIGATION OF THE EFFECT OF CD28 ON IL-2-REGULATORY TRANSCRIPTION FACTORS

Having established the requirement for CD28 stimulation in the activation of T cells it was decided to next investigate the mechanisms by which signals derived from the costimulatory pathway could enhance IL-2 transcription. Since IL-2 production is regulated via the binding of transcription factors to the promoter region 5' of the gene start site it was proposed that any direct effects of CD28 signalling would involve effects on transcription factor regulation.

4.0 USE OF CAT AND LUCIFERASE CONSTRUCTS

The use of reporter constructs enables the attachment of multiple copies of a transcription factor binding sequence upstream of the gene coding for a stable enzyme product. This provides a system whereby transcription of the enzyme is dependent upon induction of functional transcription factor complexes. The role of CD28 in the induction and functional activation of individual transcriptional factors could thus be assessed independently of and in combination with TCR stimuli. Two reporter construct systems are commonly used, those being the assessment of chloramphenicol acetyltransferase activity (CAT assays) and the detection of luciferase activity.

To efficiently perform transfection of the reporter constructs into the cells, optimal electroporation conditions, with respect to the buffer, voltage and time constant, were determined. These were initially calculated for Jurkat cells with the intention of using T cell blasts when the assay was established. Cells were electroporated over a range of conditions, dual stained with FDA as a viability marker, and PI, as a permeabilisation marker and then analysed by FACS. The results obtained from the FACS analysis are summarised in Table (IV) overleaf and are shown diagramatically in figure 4.0A .

A titration of electroporation voltage is shown in figure 4.0B and demonstrates that using 500μF, 200V was inefficient for permeabilisation and 400V resulted in a high level of cell death whereas 300V appeared optimal. In addition the effect of varying the capacitance was investigated and is shown in figure 4.0C. If the voltage was fixed at 300V, 500μF then gave optimal results.

Whilst the voltage and the capacitance were the main two factors requiring optimisation, variation of the buffer and volume of cells electroporated also affected the duration of the pulse. A greater viability was observed in the electroporated cells using Ca^{2+}/Mg^{2+} PBS compared to HeBS and small volumes resulted in a longer time constant whilst larger volumes decreased the time constant. Efficient transfection of the cells required the

identification of optimal conditions which ensured maximal permeabilisation whilst retaining cell viability. These optimal values are tabulated below (Table (V)) and shown diagramatically in the centre boxes of figures 4.0B and C.

Table IV A summary of the FACS analysis of FDA (viability) and PI (permeabilisation) staining for the electroporated cells

QUADRANT	FDA STAINING - LIVE CELLS	PI STAINING - PERMEABILISED	CONCLUSION
LOWER LEFT	—	—	Dead cells - no permeabilisation
UPPER LEFT	—	+	Cells died after permeabilisation
LOWER RIGHT	+	—	Live cells - no permeabilisation
UPPER RIGHT	+	+	Live cells which are permeabilised

Table V- Optimal electroporation conditions for the transfection of Jurkat cells

No. Cells	Volume (µl)	Voltage (V)	Capacitance (µF)	Time Const. (ms)	% of cells transfected
$2.5x10^6$	500	300	500	~ 15	20

Once the conditions had been optimised Jurkat cells were transfected with constitutive CAT constructs, the intention being to optimise the detection assay before transfecting the transcription factor-regulated CAT constructs into the cells. Figure 4.1A shows the effect of electroporation voltage on CAT activity plotted as a factor of time. Although 400V resulted in greater enzyme activity, 300V was retained as standard value as this resulted in adequate detectable enzyme activity whilst maintaining cell viability. As a large number of cells were required to enable investigation of different stimulation conditions the number of viable cells obtained from the transfection was more important than maximal activity. For the capacitance titration (figure 4.1B), the suboptimal value of 500µF was again retained to preserve cell viability. Enzyme activity increased linearly over time during the assay, the maximum reached varying depending upon the volume of extract analysed. Below 10µl ($5x10^5$ cell equivalents) an approximate linear correlation applied, however, above this the substrate was limiting resulting in a plateau by 25µl ($1.25x10^6$ cell equivalents) (Figure 4.2).

Having optimised the electroporation conditions to ensure efficient transfection, attempts were made to establish assays using the transcription factor-regulated CAT reporter constructs. Unfortunately, stimulation of Jurkat cells transfected with the constructs regulated by multiple NFAT or AP-1 transcription factor binding sites did not result in any detectable CAT activity. This may have been due to faults or mutations in the plasmid constructs or due to very low levels of enzyme induction which could not be detected. The CAT assays were therefore exchanged in favour of luciferase assays using different plasmids and a more sensitive detection method.

Luciferase reporter constructs comprising quadruple NFAT or AP-1 binding sites upstream of the luciferase gene were transfected into Jurkat cells using the optimised electroporation conditions. Figure 4.3 shows the activity induced in cells stimulated with PMA alone (0.3 ng/ml) or in combination with ionomycin (1μM). Activity is expressed as a relative value compared to control, indicating that PMA and ionomycin stimulation produced a 5 fold increase in luciferase activity compared to unstimulated samples. Interestingly, in agreement with previous reports, whilst PMA alone induced some AP-1 regulated activity (figure 4.3A), both PMA and ionomycin were required for NFAT production (figure 4.3B). This is in agreement with previous reports that a calcium signal is required for NFAT induction and implies that due to its requirement for two signals NFAT generation may be more tightly regulated than AP-1. Unfortunately attempts to use more physiological stimuli such as αCD3 and B7 transfectants resulted in only a slight increase in AP-1 regulated activity and did not induce any NFAT activity. This was in keeping with the absence of IL-2 production in Jurkat cells using these stimuli (figure 4.4).

Whilst several attempts were made to optimise the electroporation conditions required for the transfection of the blasts the viability of the electroporated cells was insufficient to enable equivalent experiments to be carried out, therefore other methods of *in vitro* transcription factor detection were investigated.

4.1 THE ELECTROMOBILITY GEL SHIFT ASSAY (EMSA)

The electromobility gel shift assay (EMSA) is an extremely useful technique which enables the visualisation and identification of individual inducible and constitutive nuclear transcription factors whilst a DNase 1 footprinting assay identifies the location of transcription factor binding within a promoter region and allows for interaction and cooperative binding between adjacent factors. Both the techniques involve the preparation of nuclear cell extracts from cell cultures and binding reactions enabling the association of the transcription factors with their respective DNA binding sites.

The EMSA was utilised in this study to investigate the role of CD28 in the induction of three transcription factors which are involved in the regulation of IL-2 gene expression. Oligonucleotides coding the binding sites for NFAT, AP-1 and NFκB were used to detect the presence of these transcription factor proteins in extracts from variously stimulated cells. Although the methodology for the assay has been fairly well documented the assay conditions required optimisation. The stimulation of different cell types results in varying levels of transcription factor induction and therefore varying quantities of protein extract are required for visualisation. In addition, variation in $MgCl_2$ concentration can alter the binding affinity of the complexes whilst the concentration of the non specific DNA, poly dI-dC affects the intensity and clarity of the signal. Therefore prior to analysing transcription factor induction using physiological stimuli a series of experiments were first carried out to optimise the EMSA .

The protein concentration of the nuclear extracts prepared from the stimulated cells was routinely measured using the Biorad assay system, calibrated using a BSA standard curve as shown in figure 4.5. Figure 4.6A demonstrates the effect of protein concentration on the detection of NFAT in T cell blasts. Compared to the oligonucleotide titration in figure 4.6B it can be seen that protein was limiting in the binding reaction. For the majority of the assays 10μg of Jurkat extract and 15μg of extract from T cell blasts proved to be sufficient to give a strong detectable signal on an overnight exposure of the autoradiograph. Excess or overloading of the extract resulted in smeared bands and eclipsing of the signals in adjacent lanes. With respect to the amount of oligonucleotide, an optimum of 40000cpm per lane which corresponded to 0.3-0.5ng was deemed to give an appropriate signal. The unbound oligonucleotide could be observed at the bottom of the gel and acted as an internal control for the activity of the oligonucleotide. Since activity was assessed by total counts, loss of label or degradation of the oligo could only be observed visually from its appearance on the gel. Activity of the oligos were assessed before each assay and adjusted accordingly to give the same strength of signal. Labelled oligos typically lasted three weeks before the ^{32}P decay decreased the activity to unusable levels.

The effect of $MgCl_2$ concentration on the binding affinity of NFAT in T cell blasts was investigated and is shown in figure 4.7A. Although the observable difference was small in this experiment an optimum concentration of 0.6mM was found to produce consistent binding for all the transcription factors studied.

Poly dI-dC is double stranded DNA with a repetitive inosine/ cytidine sequence. The inclusion of this reagent in the binding mix serves to increase the specificity of the detected proteins by providing a binding environment for non-specific DNA binding proteins. Due to the high concentration of poly dI-dC in the mix non-specific proteins

preferentially bound this DNA whilst the transcription factors which had a higher affinity for their respective oligonucleotide sites bound preferentially to the labelled DNA. In the absence of poly dI-dC large non-specific protein complexes bound the oligo forming an aggregate which did not enter the gel. However, if excess poly dI-dC was added, even the proteins of interest bound to the poly dI-dC and no signal was observed. A titration of poly dI-dC concentration was therefore performed (figure 4.7B) and an optimal concentration of 1μg / reaction was found to result in a strong signal, which appeared to be specific to the oligo of interest.

Despite the inclusion of poly dI-dC, some proteins, other than the relevant transcription factor, do bind to the oligonucleotides. However, the specificity of the complexes observed in the presence of the optimal poly dI-dC concentration can be confirmed by cold competition reactions. These involve the addition of 100 fold excess of unlabelled oligo of the relevant and an irrelevant binding site to different control tubes. Disappearance of the binding complex in the presence of the relevant oligo and not the irrelevant oligo was taken to indicate specific binding. As shown in figure 4.8, cold competition of the NFAT complex using excess unlabelled NFAT oligo resulted in a loss of detectable binding whereas an excess of NFκB oligo had no effect on the complex. This would not have been the case if the protein was binding non specifically to the DNA. It was also observed, however, that the NFAT complex was partially competed by excess AP-1 oligo. This was due to the presence of AP-1 components in the NFAT complex, namely fos and jun family members, which resulted in some cross reactivity between the two oligos. The AP-1 oligo could therefore not be regarded as an entirely irrelevant DNA sequence.

The majority of transcription factor studies reported previously were carried out using mitogenic stimulation of Jurkat cells (PMA and ionomycin or PMA and PHA). Whilst this gave valuable information on the presence and inducibility of the complexes in T cells it did not confirm their induction under physiological conditions. Furthermore the signalling pathways active in Jurkat cells have been shown to be somewhat altered from normal T cells in that mitogenic stimulation caused a downregulation in their proliferative state although IL-2 production was significantly increased. Thus PMA and ionomycin were very effective control stimuli for the induction of IL-2 regulatory transcription factors in Jurkat cells and were used initially to establish the detection and inducibility of the complexes in the cell extracts in order to validate the assay before investigating the role of CD28. Figure 4.9A shows the inducibility of the three transcription factor complexes in Jurkat cell extracts. Although due to the constitutively activated phenotype of these cells a small amount of constitutive NFAT and AP-1 transcription factor expression was detectable, the levels of all three factors were considerably increased following stimulation of the cells. The upper bands

in each case represent the specific transcription factor although each factor has a different mobility in the gel due to differences in complex size.

Subsequent examination of the PMA/ ionomycin T cell blast extracts revealed that even at 8 days after their initial stimulation the basal levels of transcription factors were maximal (figure 4.10) and were therefore not visibly upregulated on restimulation. Even at a later time point basal levels remained too high to detect further induction. It was therefore not possible to use these blasts for the detection of transcription factor induction.

The high basal expression of the IL-2 regulatory transcription factors indicated an activated cell state which was in agreement with the high basal proliferation observed in the initial experiments. However, whilst transcription factor expression appeared to be maximal, no IL-2 production had previously been detected in these day 8 unstimulated cells indicating an insufficient level of activation to induce IL-2 transcription. However, since B7-induced signals had been shown to costimulate suboptimal PMA and ionomycin activation, the effects of B7 on these partially activated cells was investigated. Figure 4.11 shows a timecourse of the proliferative and IL-2 responses observed following B7 stimulation of the PMA/ionomycin T cell blasts in the absence of other stimuli. Although not as significant as mitogenic restimulation, B7 transfectants alone did appear capable of inducing limited proliferation and IL-2 production in these previously activated cells.

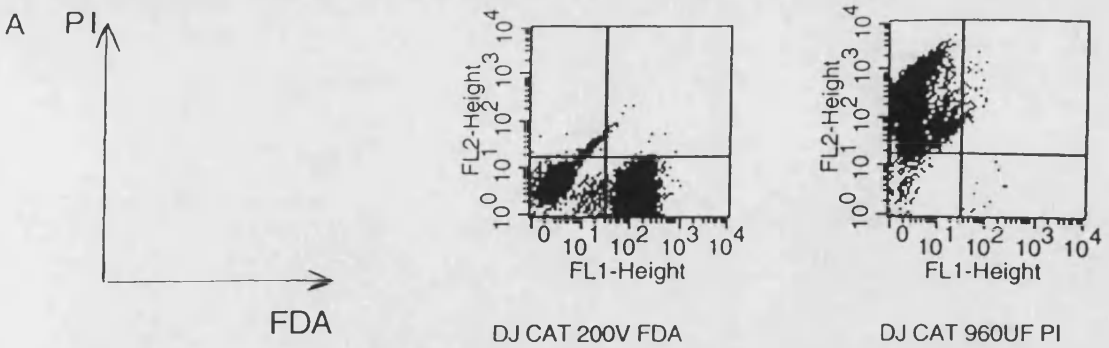
4.2 DNase 1 FOOTPRINTING

In parallel with the EMSAs, DNase digestion assays, using the same nuclear extracts, were performed on the IL-2 promoter to identify the protected transcription factor binding sites. The main aim of this was to investigate the effects of costimulation on the promoter region as a whole and it was also hoped that some information concerning the occupation of the CD28RE would be gained from such assays. Probes encoding the IL-2 gene promoter region were subcloned and then labelled at different ends so that the footprint could be analysed from either end of the promoter sequence. After extensive optimisation of the conditions a footprint was obtained for the 5' end of the promoter distal to the start site. As is shown in figure 4.12 whilst incubation of the DNA with unstimulated Jurkat cell extracts resulted in full digestion of the DNA, in the presence of stimulated nuclear extracts some regions were apparently protected from digestion by the binding of transcription factors. The region shown covers the NFAT and OCT binding sites which were protected in the presence of extracts from PMA and ionomycin stimulated Jurkat cells indicating the presence of bound protein complexes. Unfortunately the resolution of the gel was insufficient to detect the downstream

CD28RE which would have required the synthesis of a new probe nearer to the site of interest but there was insufficient time available to continue this study. The assay was not carried out using the T cell blasts as an unstimulated control was needed to differentiate between transcription factor protected DNA regions and unprotected, incompletely digested regions.

4.3 SUMMARY

The reporter construct assays, EMSA and DNAse footprinting were all successfully established using mitogenically activated Jurkat cells. However, investigation of transcription factor induction in the T cell blasts proved to be more complicated. Transfection of the T cell blasts was not possible using electroporation methods due to the extremely low viability of the permeabilised cells. Furthermore transcription factor analysis in the T cell blasts revealed that despite additional signals being required to stimulate proliferation and IL-2 production the original PMA/ionomycin stimulus had a prolonged effect on the T cell blasts resulting in constituitve transcription factor expression. The effects of CD28 costimulation on transcription factor induction could thus not be assessed in these cells.



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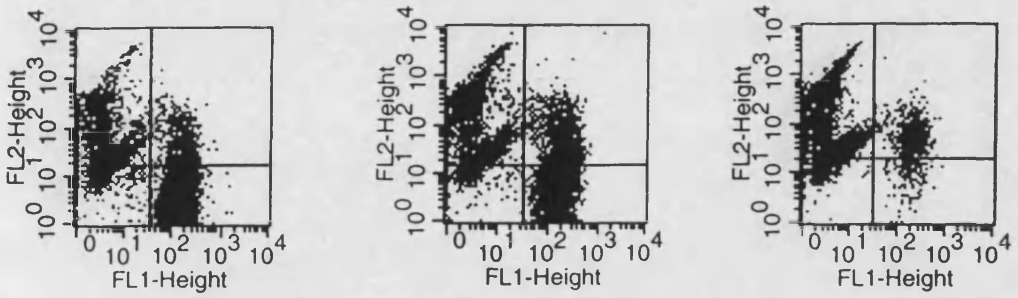
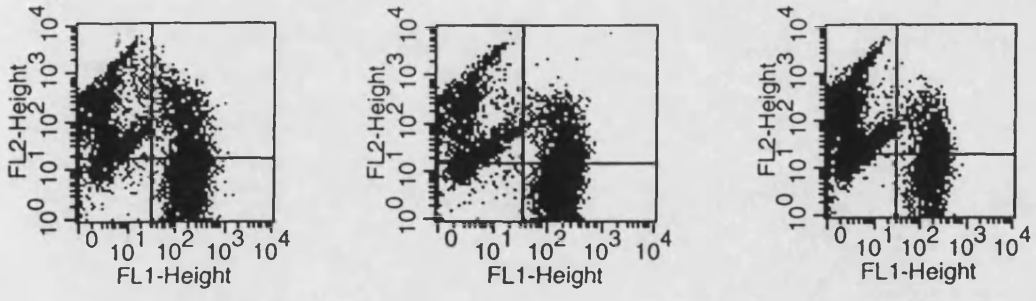


Quad	% Total
UL	23.09
UR	8.61
LL	9.21
LR	59.09

Quad	% Total
UL	26.00
UR	24.84
LL	6.42
LR	42.74

Quad	% Total
UL	60.35
UR	11.70
LL	24.70
LR	3.25

C



Quad	% Total
UL	29.78
UR	18.57
LL	8.41
LR	43.24

Quad	% Total
UL	26.04
UR	24.80
LL	6.46
LR	42.70

Quad	% Total
UL	48.78
UR	11.64
LL	21.73
LR	17.85

Figure 4.0 Optimisation of the electroporation conditions for Jurkat cells

Panel (A) shows the control FDA and PI staining. Jurkat cells were then electroporated using varying voltage (B) and capacitance (C) values. The cells were then immediately stained with FDA (0.1ng/ml) and PI (4µg/ml) before being FACS analysed.

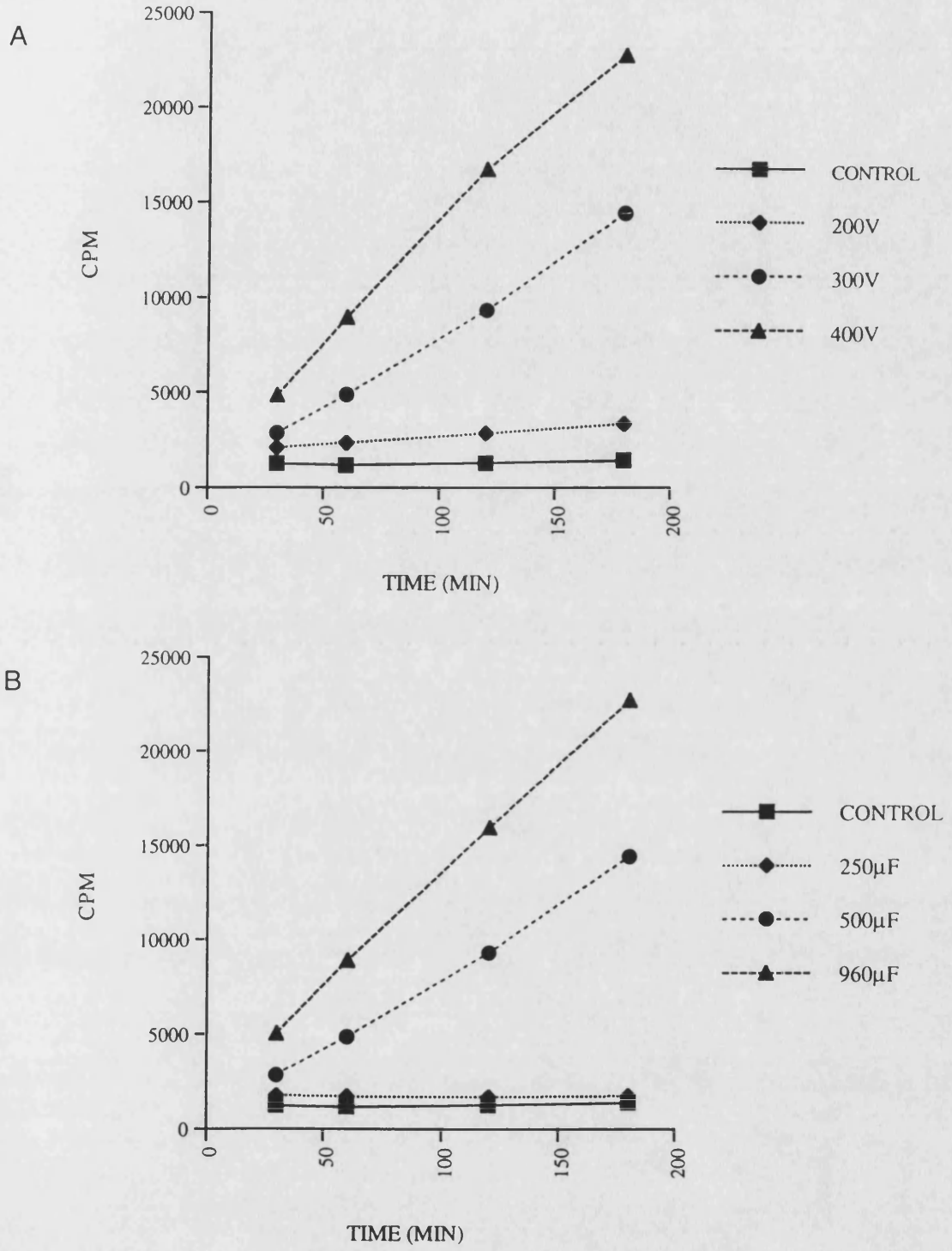


Figure 4.1 Effect of electroporation conditions on constitutive CAT activity in Jurkat cells

Jurkat cells were transfected with constitutively activated CAT reporter constructs (10µg) using varying voltage (A) and capacitance (B) electroporation values. CAT activity was assessed *in vitro* using cytoplasmic cell extracts 48 hours later and is plotted as a factor of time.

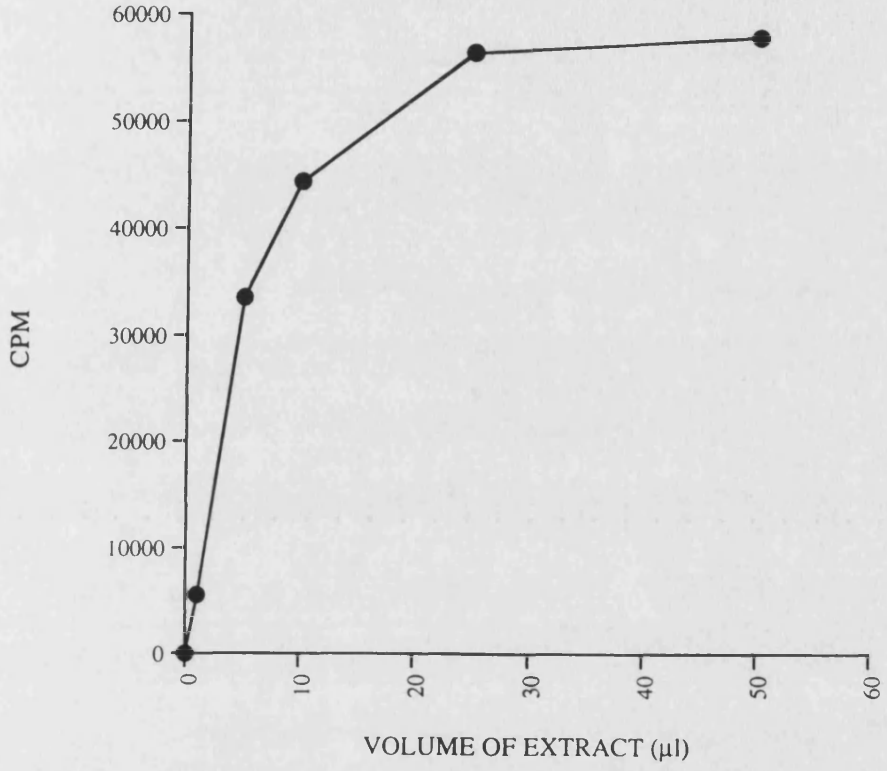


Figure 4.2 Effect of extract volume on the measurement of constitutive CAT activity in Jurkat cells

Jurkat cells were transfected with constitutively activated CAT reporter constructs and cytoplasmic cell extracts were prepared 48 hours later. CAT activity was then assessed in increasing volumes of cell extract to determine the linearity range of the assay.

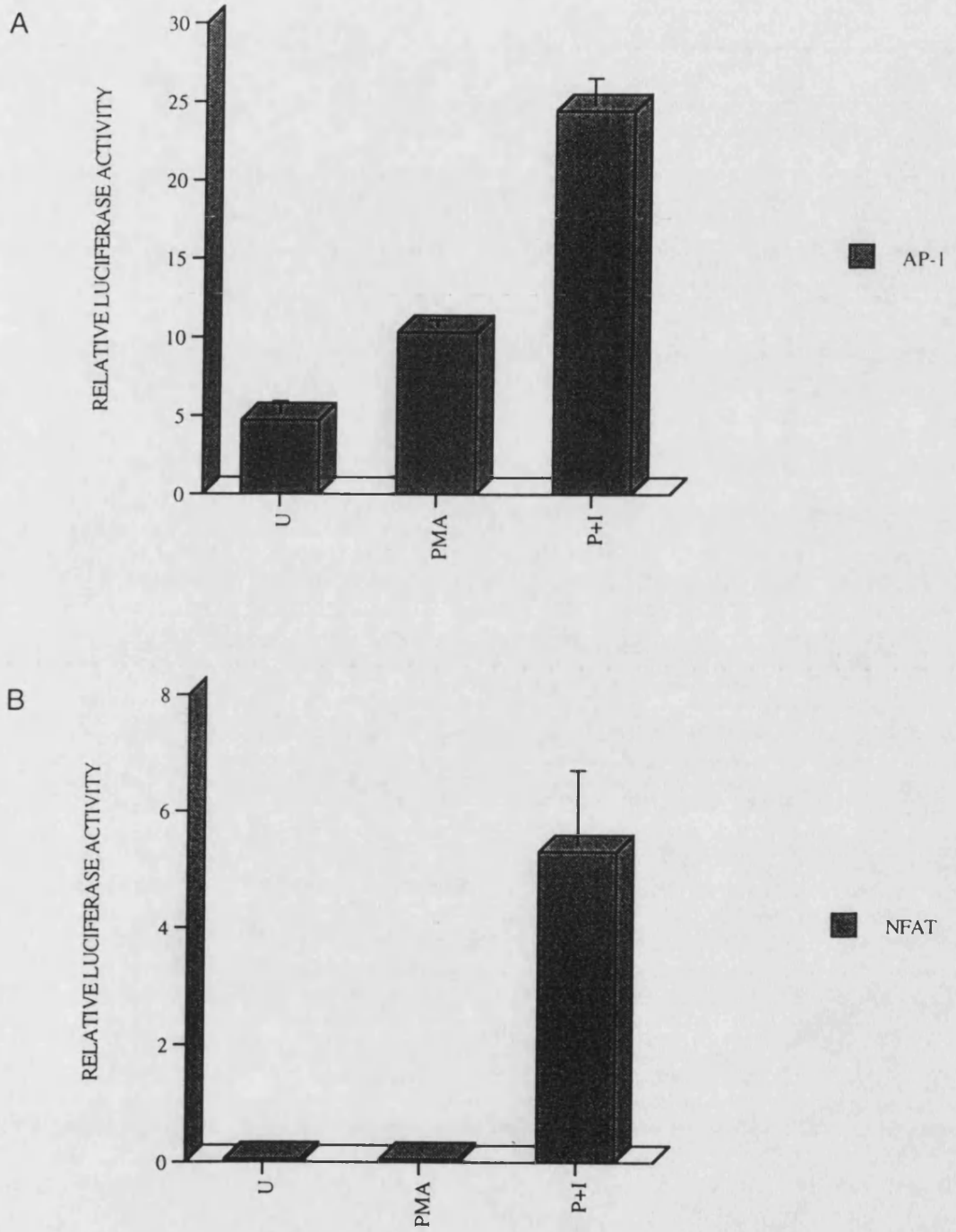


Figure 4.3 Assessment of transcription factor regulated luciferase activity in mitogen stimulated Jurkat cells

Jurkat cells were transfected with luciferase constructs regulated by quadruple AP-1 (A) or NFAT (B) transcription factor binding sites. 24 hours later the cells were washed and placed in media, media containing PMA (0.3ng/ml) or media with PMA and ionomycin (1μM). After 4 hours cell extracts were prepared and assayed for luciferase activity. Data are the mean of three assays.

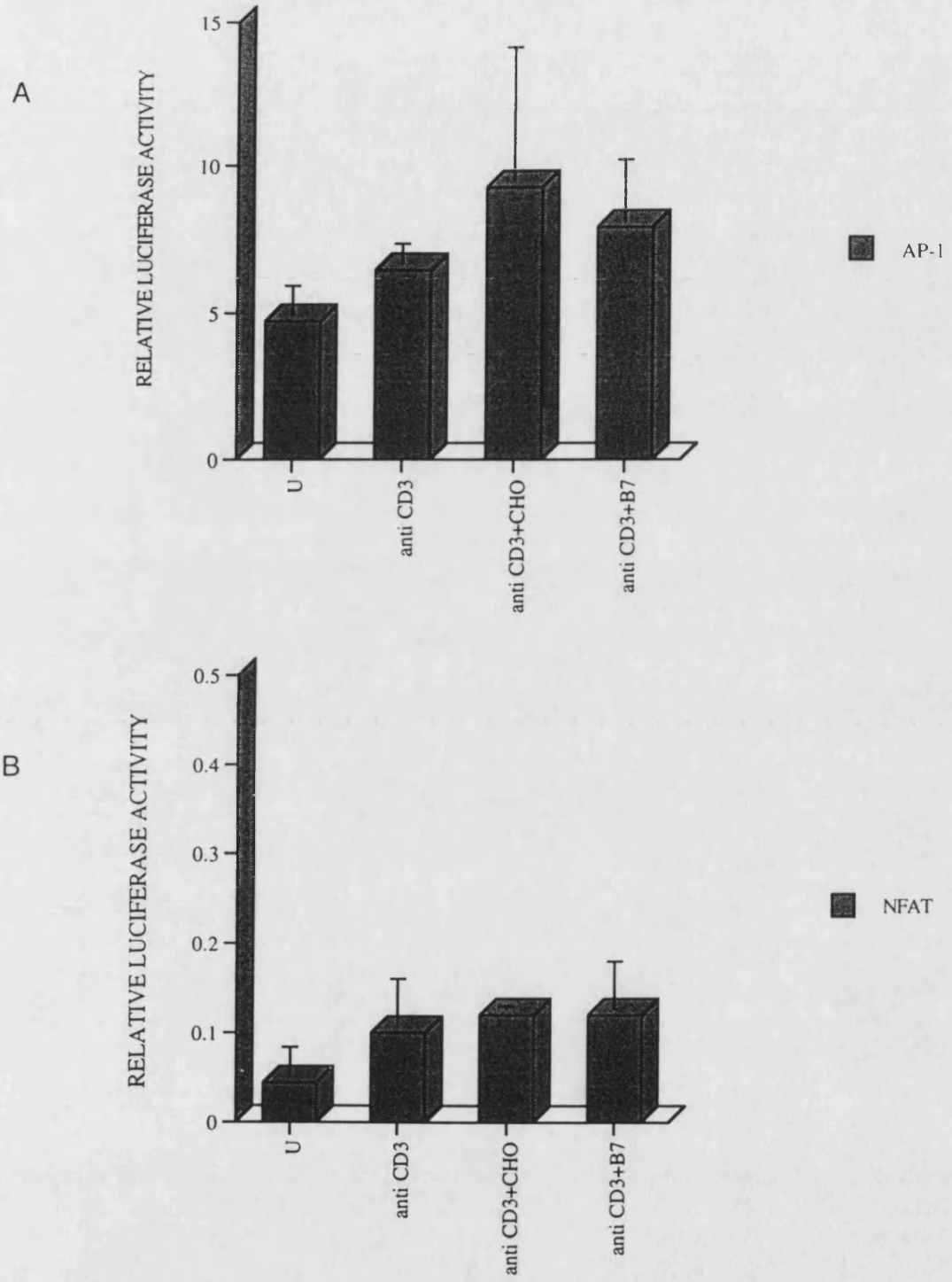


Figure 4.4 Assessment of transcription factor regulated luciferase activity in stimulated Jurkat cells

Jurkat cells were transfected with luciferase constructs regulated by quadruple AP-1 (A) or NFAT (B) transcription factor binding sites. 24 hours later the cells were washed and left unstimulated or activated with cross- linked anti CD3 (1μg/ml) alone or in the presence of fixed CHO cells or CHO-B7 cells. After 4 hours cell extracts were prepared and assayed for luciferase activity. Data are the mean of three assays.

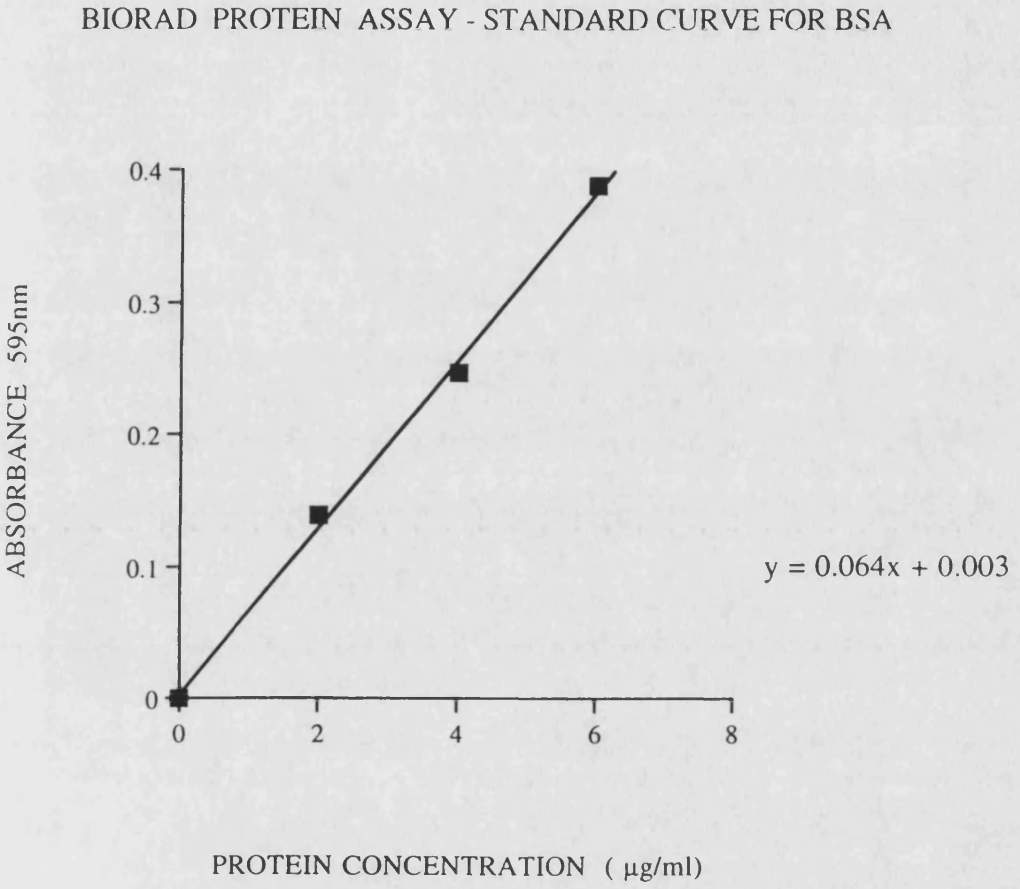


Figure 4.5 Biorad protein assay - standard curve

A standard curve of absorbance at 595 nm for serial dilutions of BSA was produced to enable calibration of the assay for the assessment of nuclear extract protein content.

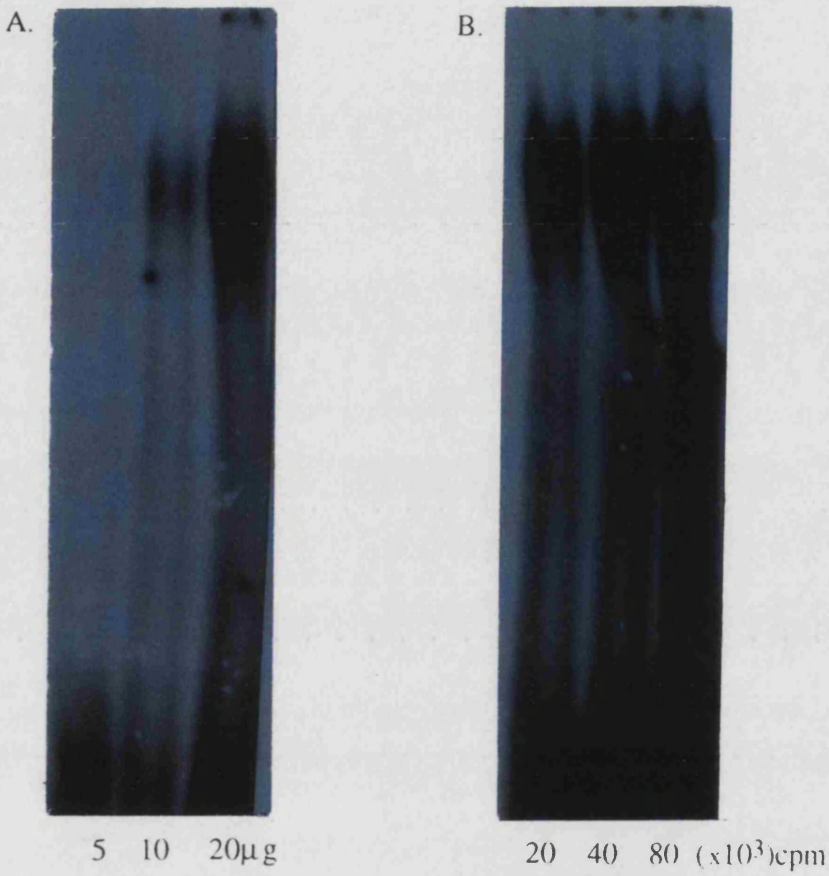


Figure 4.6 Effect of protein and oligonucleotide on the detection of transcription factor complexes

Nuclear extracts were prepared from T cell blasts and were analysed for NFAT induction. Varying concentrations of nuclear extract protein (A) or labelled oligonucleotide (B) were included in the binding reaction and gel analysis performed as stated in the protocol. Complexes were visualised by autoradiography following an overnight exposure of the gel.

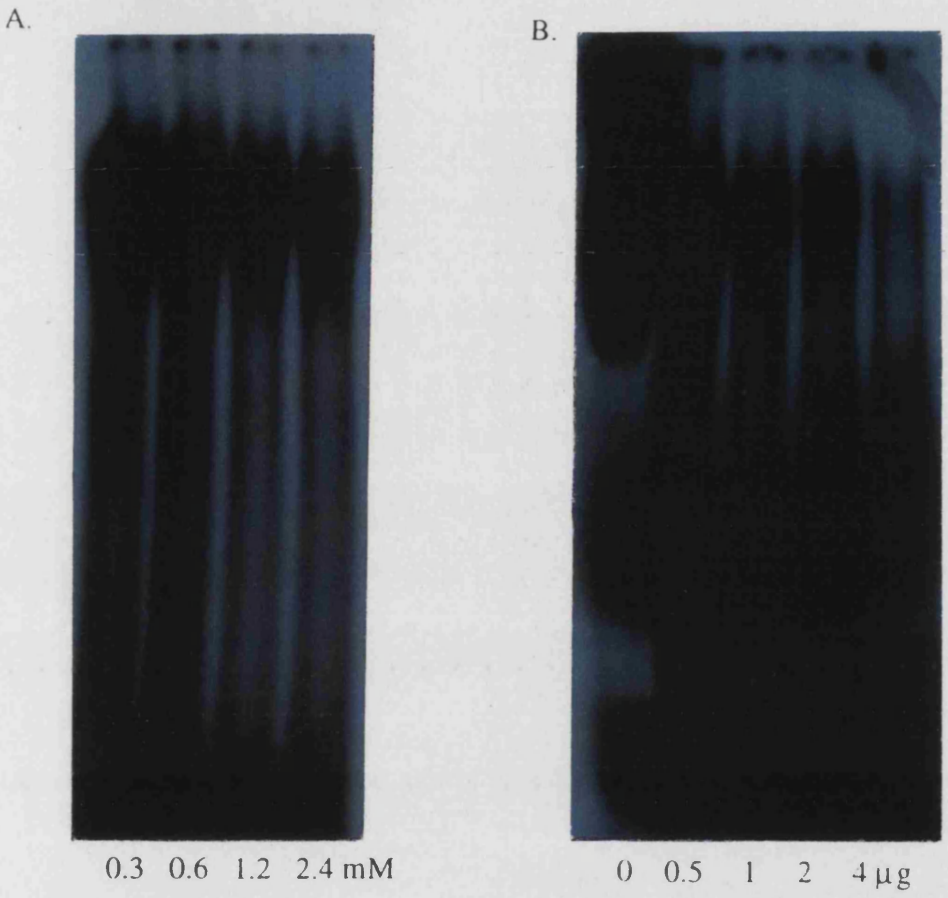


Figure 4.7 Effect of $MgCl_2$ and poly dI-dC concentration on the detection of transcription factor complexes

Nuclear extracts were prepared from T cell blasts and were analysed for NFAT induction. Varying concentrations of $MgCl_2$ (A) or poly dI- dC (B) were included in the binding reaction and gel analysis performed as stated in the protocol. Complexes were visualised by autoradiography following an overnight exposure of the gel.

Figure 4.8 Confirmation of transcription factor complex specificity by cold competition

Nuclear extracts were prepared from PMA (0.3ng/ml) / ionomycin (1μM) stimulated t cell blasts.These were analysed for NFAT complexes in the absence (--) and presence (NFAT (N), AP-1 (A), NFκB (K)) of 100 fold excess of cold oligonucleotide. Complexes were visualised by autoradiography following an overnight exposure of the gel.

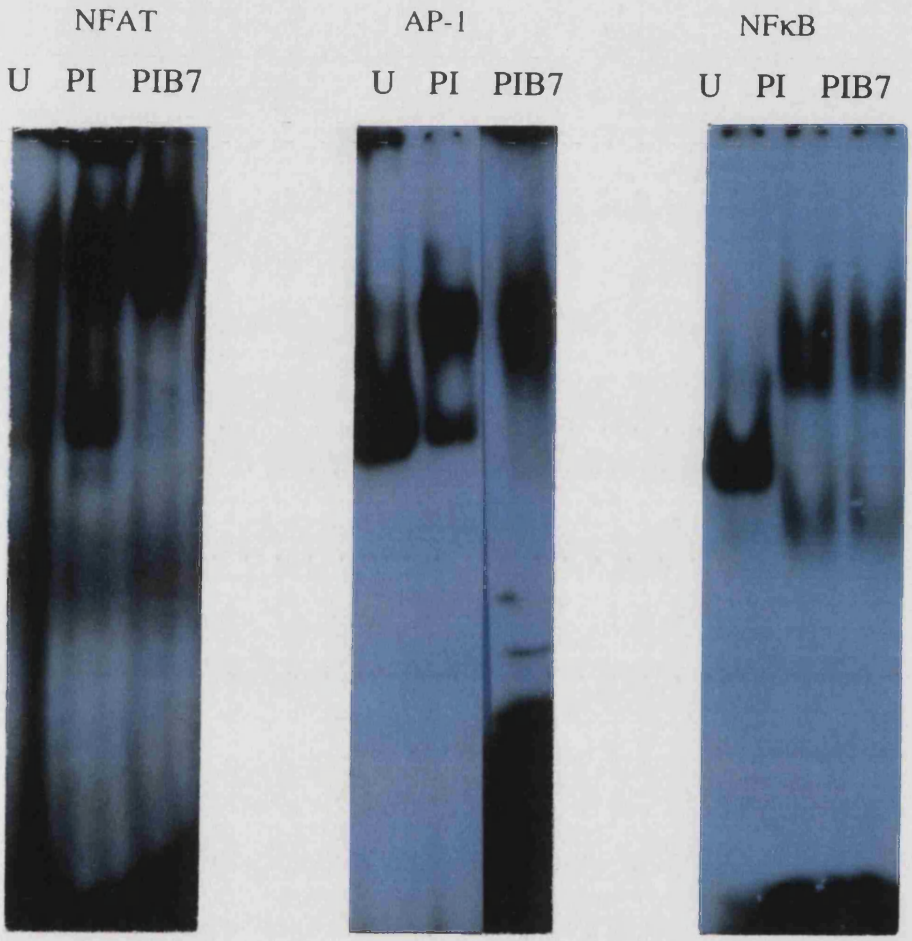


Figure 4.9 Induction of transcription factor complexes

Nuclear extracts were prepared from unstimulated (U) ,PMA (0.3ng/ml) / ionomycin (1μM) and PMA/ionomycin /B7 (PIB7) stimulated Jurkat cells and were analysed for the production of NFAT, AP-1 and NFκB complexes. These were visualised by autoradiography following an overnight exposure of the gel.

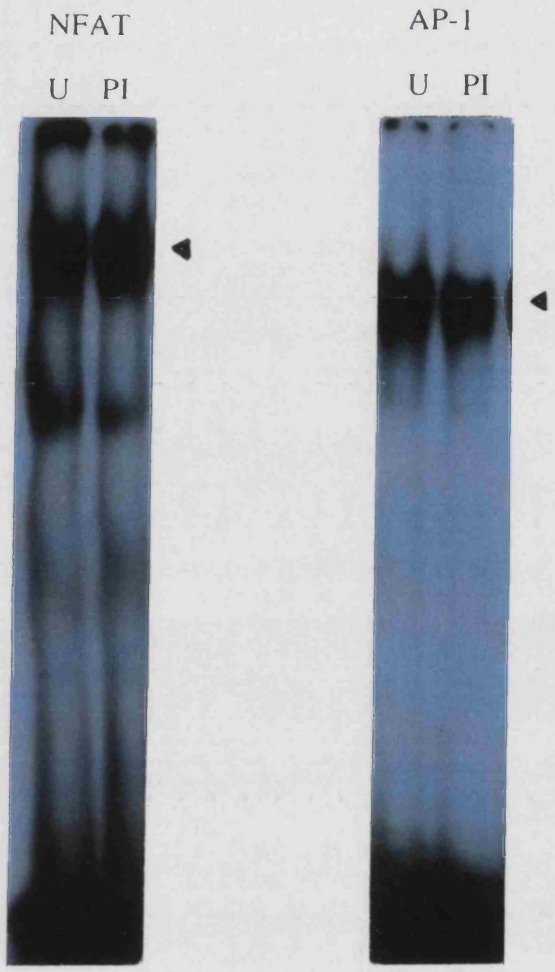


Figure 4.10 Induction of transcription factor complexes

Nuclear extracts were prepared from unstimulated (U) and PMA (0.3ng/ml) / ionomycin (1μM) stimulated T cell blasts and were analysed for the production of NFAT and AP-1 complexes.These were visualised by autoradiography following an overnight exposure of the gel.

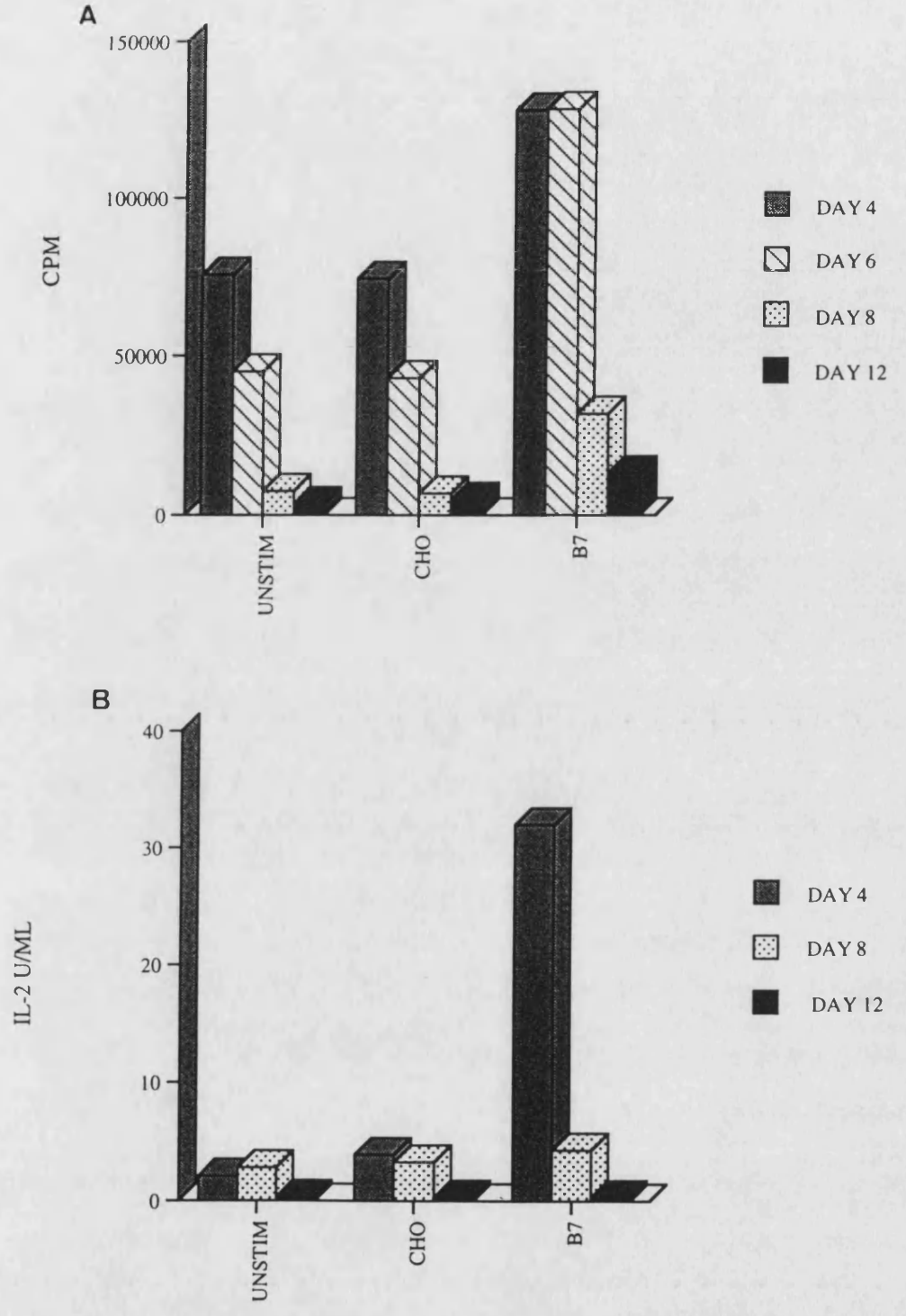


Figure 4.11 Effect of B7 stimulation on proliferation and IL-2 production in PMA/ ionomycin T cell blasts

PMA/ ionomycin T cell blasts were taken from culture on the day specified after restimulation and were incubated in new media alone or in the presence of fixed CHO cells or fixed B7 cells for 24 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using the CTLL bioassay. Data are the triplicate mean of a representative experiment.

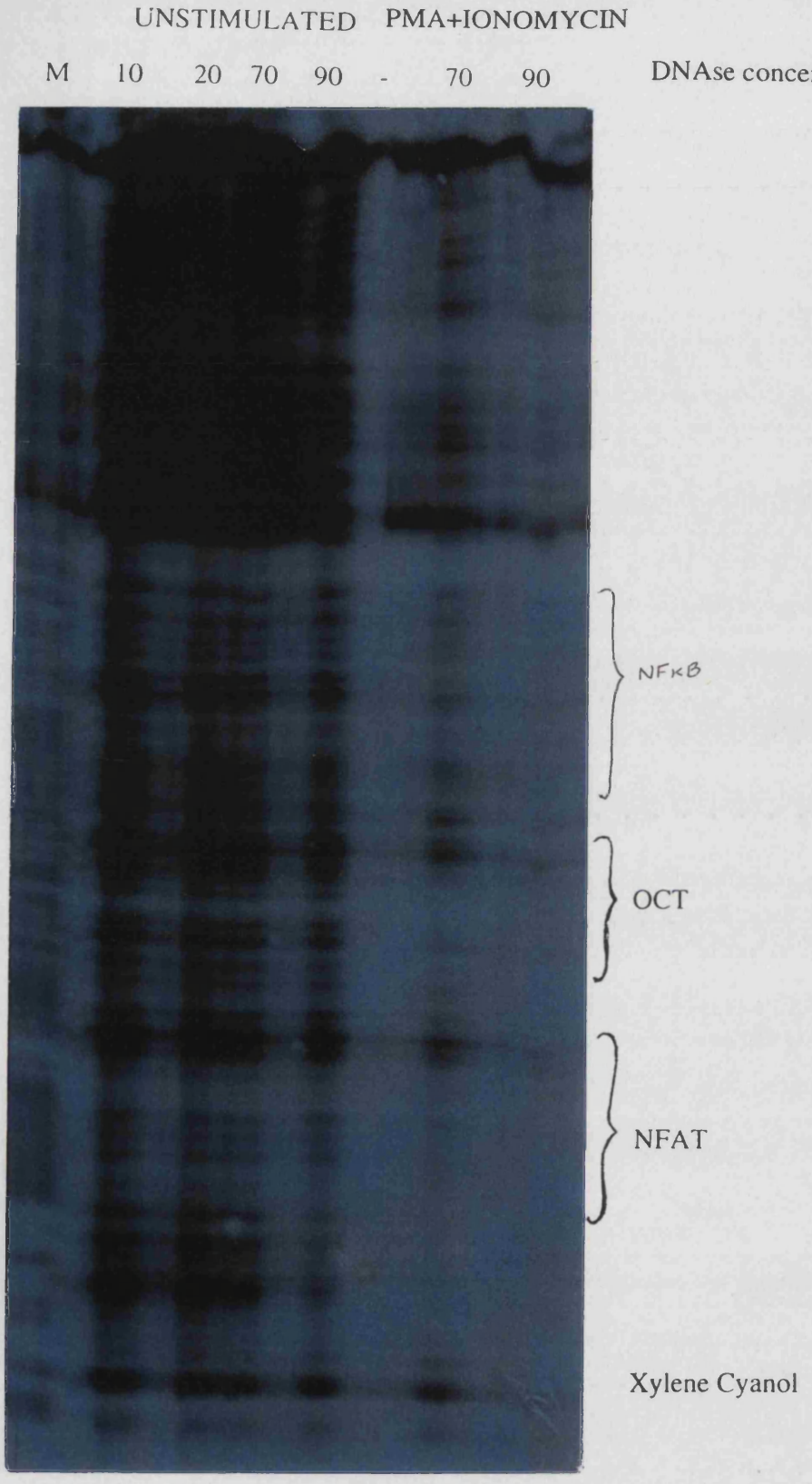


Figure 4.12 DNAse 1 Footprinting of the IL-2 promoter

DNAse 1 digestion of the distal end of the IL-2 promoter was assessed in the presence of nuclear extracts from unstimulated and PMA/ionomycin stimulated Jurkat cells using a range of DNase 1 enzyme concentrations.

4.4 DISCUSSION

Whilst functional whole cell assays provide valuable information on the response of a cell to a certain stimulus they do not give any insight into the intracellular targets of the signals derived from stimulation of the surface receptors. Neither is it possible to determine the intracellular effects of the costimulatory signals as distinct from those of the TCR signals. By studying the regulation of transcription factors it was hoped that it may be possible to identify some downstream targets of the CD28 signalling pathway. Previous reports have indicated that CD28 signals regulate the IL-2 promoter (Lindsten et al., 1989a; Lai et al., 1995; Umlauf et al., 1995) although it is still not known to what extent this is due to mRNA stabilisation as opposed to direct effects on transcription factor induction. Data obtained from my initial studies indicated that CD28 stimulation in the presence of PMA could upregulate IL-2 production and I therefore sought to establish whether this response was mediated by a direct effect upon transcription factor induction or activation. Several different qualitative and functional assays were established to study the effects of CD28 costimulation on IL-2 - associated transcription factor induction.

Despite the observed differences between immortal cell lines such as Jurkat cells and resting T cells such as their activated phenotype and IL-2 independence Jurkat cells are still frequently used to investigate CD28 signalling mechanisms and IL-2 regulation. Furthermore as several groups have already shown that many of the IL-2 regulatory transcription factors can be induced in Jurkat cells using PMA in combination with a secondary stimulus (Banerji et al., 1991; Durand et al., 1988; Shaw et al., 1988) these cells provided a convenient positive control in the establishment of such assays.

Initially a functional assay was developed involving transcription factor-regulated reporter constructs as there are several advantages associated with measuring enzyme induction rather than cytokine output. Whilst the CTLL bioassay gives a reasonable assessment of IL-2 output it relies on effective translation of the IL-2 message and does not measure receptor bound or internalised IL-2, hence the final reading is only an estimate of cytokine production. Reporter constructs allow the assessment of induction of individual transcription factors and enable measurement of total enzyme induction within the cell, unhampered by translation, secretion or reuptake mechanisms.

The electroporation of Jurkat cells proved to be a very effective method for the transfection of reporter constructs. However, whilst electroporation is an extremely useful technique the optimal conditions vary considerably depending upon the cell type. In agreement with other groups, I found transfection of transformed cells considerably more successful than transfection of normal T cells (personal communications). The latter required harsher electroporation conditions to permeabilise the membranes and

consequently the cells exhibited poor survival capabilities. Furthermore transformed cells are often larger than normal cells thus membrane transfer of plasmid DNA is less of a cell stress factor. Preliminary attempts to transfect the T cell blasts using lipid mediated transfer techniques (liposomes) (Mannino and Gould-Fogerite, 1988) were unfortunately also unsuccessful so alternative methods of transfection such as DEAE-Dextran (Durand et al., 1987) or calcium phosphate(Sambrook et al., 1989) may be required.

A comparison of the results obtained from the FDA/PI staining with those of the reporter construct enzyme assays showed an extremely good correlation between permeabilisation and enzyme activity indicating that the cells which were permeabilised took up the reporter plasmid very efficiently. Thus the electroporation/ staining procedure enables the optimisation of electroporation conditions prior to the introduction of the DNA and could be used for any transfection to ensure the maximum efficiency of transfections whilst maintaining cell viability. A further advantage is that the FDA/ PI staining provides immediate results on the effectiveness of the electroporation and requires very few cells to optimise the conditions .

Whilst significant induction of luciferase activity was observed in the mitogen-stimulated transfected Jurkat cells the effects of anti-CD3 stimulation were less convincing. Contrary to some reports (Ward and Cantrell, 1990) in my experience despite adequate surface expression of CD3 and CD28 on the Jurkat cell surface, activation of these receptors appeared to be very ineffective as a means of complete cellular activation although B7 stimulation of Jurkat cells could be shown to trigger some receptor proximal events as discussed in chapter 5. This therefore implies an alteration in the downstream signal transduction pathways as a result of the transformed phenotype of the cells rather than an insensitivity of the receptor itself. Overall, however, attempts to activate Jurkat cells using physiological stimuli were found to result in less significant responses than were observed using PMA and ionomycin: a further reason for developing an alternative T cell model which could respond efficiently to physiological stimuli.

Due to the inability to successfully transfect normal T cells it was necessary to establish alternative assays to detect transcription factor induction and regulation. Although the EMSA does not measure the transcriptional activity of detected complexes it does provide detailed information on the induction requirements of specific transcription factors. Despite the apparently activated phenotype of Jurkat cells, basal levels of the IL-2 regulating transcription factors are either non existent or extremely low and have been shown to be strongly induced following stimulation of the cells with PMA and ionomycin. Furthermore, the induction of the transcription factors correlates well with the induction of IL-2 production indicating that despite the other abnormal responses to PMA demonstrated by these cells the IL-2 promoter is still functional. Unfortunately the

addition of B7 appeared to have little further effect upon the expression of the transcription factor complex indicating that the PMA/ ionomycin stimulus was maximal for the induction. Although there are no published reports of transcription factor induction in B7 stimulated Jurkat cells several groups have shown increased activity following the addition of αCD28 antibodies to PMA and iomnomycin stimulated T cells (Fraser et al., 1991; Ghosh et al., 1993). However, these studies were investigating the induction of the CD28RC which may be more directly dependent upon CD28 mediated signals that the other IL-2 regulatory transcription factors. Furthermore the nature of the signals delivered by antibody stimulation of the CD28 receptor has been shown to vary depending upon the state of cross-linking of the antibody (Ledbetter et al., 1990) therefore it is difficult to compare the antibody responses with transfected ligand. Despite using suboptimal PMA concentrations to enable detection of the effects of a costimulatory signal, the concentration of PMA present still appeared adequate for the full induction of the transcription factors. However, the previous experiments showing a comparison of the amount of IL-2 produced using this suboptimal concentration of PMA in the presence and absence of B7 costimulation revealed a significant increase in output with B7. Thus the inclusion of a B7 stimulus may enhance transcription factor production but it is not easily observed in this assay. Alternatively the B7 induced effect on IL-2 output may be due to its effects on mRNA stabilisation and additional assays to measure IL-2 mRNA would be required to determine the exact mechanism of the B7 induced upregulation of IL-2.

Whilst information on transcription factor inducibility was obtained from these Jurkat cell assays the main aim of studying transcription factor induction in the T cell blasts activated using PMA and ionomycin proved not to be possible. The initial stimulus used to prepare the T cell blasts appeared to have a prolonged effect on their activation state and transcription factor analysis revealed high levels of expression in both the unstimulated and PMA/ ionomycin stimulated extracts. Comparison of these data with the proliferation and IL-2 assays revealed that whilst unstimulated day 8 cells had a slightly raised basal proliferation no IL-2 was produced and yet transcription factor expression appeared to be maximal. This indicated that the complexes present were either insufficient to bridge a threshold required for IL-2 transcription or that the complexes were not transcriptionally active. Previous reports have indicated that whilst PMA can induce transcription factor expression additional signals are required to functionally activate the complexes (Rincon and Flavell, 1994). Since stimulation of these cells with B7 alone at early time pointsdid result in some IL-2 output this indicated that one role of the CD28 costimulatory signal may be the post translational modification of the PMA-induced complexes. Therefore although the cells only received one dose of PMA 8 days prior to the transcription factor assay sufficient levels of PMA remained in the media to keep the cells in a partially activated state from which they required a

costimulation to upregulate proliferation and induce IL-2 production. Washing the cells 24 hours prior to experimental use still revealed an activated phenotype indicating that PMA stimulation had a prolonged effect on the activation of the intracellular signalling pathways.

Unfortunately although it was possible to establish the technique of DNAse 1 footprinting the preliminary assays did not yield much significant new information. The detection of adjacent NFAT and OCT factor binding indicated that the occupation of both of these sites was required for IL-2 transcription. Furthermore since Jurkat cells do not express Oct2 (Staudt et al., 1988) the distal Oct site is presumably bound by the Oct1 proteins. The detection of factors at both sites was in agreement with previous studies involving the IL-2 promoter (Durand et al., 1988). Of comparative interest is that whilst Pfeuffer et al (Pfeuffer et al., 1994) reported the inhibition of OCT factor binding by NFAT on the IL-4 promoter, the detection of the binding of both factors on the IL-2 promoter footprint indicates that the effect is specific to the IL-4 promoter and is not a general feature of cytokine regulation.

From the responses observed it appeared evident that the PMA and ionomycin T cell blasts, certainly with respect to transcription factor induction, represented a highly activated T cell model. Therefore although their overall activation responses at day 8 mirrored those of resting T cells it was not possible to use these cells to study the effects of costimulation on the induction of the transcription factors controlling IL-2 production. However, these assays did indicate a role for CD28 signals in the post translational modification of the complexes. Exactly what this modification involves is not yet known but may involve phosphorylation reactions or conformational changes to alter the protein/ DNA interaction.

Due to the prolonged activation effect of the initial PMA stimulus I therefore sought an alternative stimulus for the preparation of T cell blasts which hopefully would enable complete quiescence of the cells so as to observe the inducibility of the transcription factor complexes.

CHAPTER 5 - CD28 SIGNALLING IN SEA T CELL BLASTS

5.0 ESTABLISHMENT OF SEA-ACTIVATED T CELL BLASTS

Due to the prolonged activated state of the PMA/ ionomycin T cell blasts, it was not possible to study CD28-mediated effects on transcription factor regulation in this system and in light of this, other stimuli for inducing T cell blasts, including PHA and the superantigen SEA, were investigated. PHA may stimulate T cells via binding to CD2, whilst superantigens, derived from staphylococcal enterotoxins, activate T cells by cross-linking the T cell receptors with MHC class II proteins. As superantigens bind to the outside of the receptor and not within the peptide binding groove, they activate a much larger percentage of T cells than a conventional peptide antigen. Furthermore the requirement for MHC-matched APCs in the presentation of peptide antigen is removed allowing the use of DR4 transfectants as APCs. The advantages of this form of activation are that the signals produced by superantigens are transduced through the TCRα/β chains and so activate the same signalling pathways as a peptide antigen. This, coupled with the observation that superantigen-stimulated blasts were more proliferative in culture than PHA blasts, led initially to the adoption of SEA as an alternative stimulus for the preparation of T cell blasts.

5.1 PROLIFERATION PROFILE OF SEA BLASTS

As for the PMA/ ionomycin blasts, a proliferation profile showing the response to various stimuli was produced for the SEA-activated T cell blasts. Figures 5.0 to 5.2 show the effects of stimulation on the proliferation and IL-2 production of SEA blasts, taken 8 days after restimulation with antigen. At this timepoint the cells had a very low basal proliferation rate and did not produce any detectable IL-2. Restimulation of the blasts with SEA antigen presented on CHO-DR4 transfectant cells resulted in a dramatic increase in proliferation and significant IL-2 output as shown in figure 5.0. The cotransfection of B7 had only a slight additional effect on proliferation but increased IL-2 output by a further 2.5 fold.

Stimulation of the Day 8 SEA blasts with a suboptimal PMA concentration (0.3ng/ml) or with ionomycin alone had no appreciable effect on either proliferation or IL-2 output of the cells, however, the combination of the two agents produced an effective synergy resulting in an approximate 25 fold increase in proliferation (figure 5.1) and a significant IL-2 output. The addition of B7 transfectants to the stimulated cells further increased proliferation but did not affect IL-2 production indicating the level already induced was maximal.

Control stimulations of the SEA-activated blasts using transfectants alone, however, revealed some interesting characteristics of these cells. Whilst the addition of fixed CHO cells to the blast cultures had no effect, the presence of B7 transfectants stimulated a 3 fold increase in proliferation but unlike the PMA/ ionomycin blasts did not induce any detectable IL-2 output (figure 5.2). Combined with the previous results this appeared to indicate that B7 alone could drive proliferation in these cells but that additional signals were required for the production of IL-2. Although not as great as that observed with DR4-SEA or PMA and ionomycin, the B7 induced proliferation was significant and highly reproducible. Furthermore it provided the opportunity to investigate the CD28-driven signalling pathway in relative isolation without interference from TCR derived signals. This response to B7 alone appeared to be specific to T cell blasts and was not observed with either the Jurkats or resting T cells.

The lack of IL-2 production following B7 stimulation of the SEA blasts was an interesting if unexpected observation since IL-2 is known to be the main T cell proliferative cytokine and stimulation with PMA and ionomycin did result in IL-2 output. Furthermore, unlike the PMA/ ionomycin blasts, investigation of the B7 induced responses at day 4 still revealed no detectable IL-2 output from the cells despite their expression of IL-2 Rα (CD25). It was proposed that either the cells were producing very low levels of IL-2 which was just sufficient for autocrine proliferation and hence rapidly internalised or that they may have been producing an alternative cytokine such as IL-4 which is also known to cause proliferation in T cells. An IL-2 ELISA was carried out on cell supernatants to check for low levels of cytokine production whilst an IL-4 ELISA was also carried out to investigate the possible production of this cytokine. The standard curves for the assays are shown in figures 5.3A and B respectively whilst the sample readings are tabulated below (Table VI). However, IL-2 was only detected in the supernatant of the PMA and ionomycin stimulated blasts whilst no IL-4 could be detected in the supernatant samples of either B7 or PMA / ionomycin stimulated blasts.

Table (VI) Cytokine concentration in sample supernatants measured by ELISA

	IL-2	IL-4
PMA+IONOMYCIN	20.7ng/ml	0
B7	0	0

In an attempt to discover whether the proliferation was indeed mediated by a soluble factor a test proliferation involving the addition of supernatant from B7-stimulated and SEA-pulsed DR4/B7 SEA blasts to unstimulated blasts was set up. Unfortunately no significant proliferation in the set of cells which received the B7-stimulated blasts supernatant was observed as shown in figure 5.4 although proliferation was observed

with the PMA/ionomycin and DR4/B7-SEA supernatants. The interpretation of this result was difficult as it could either indicate a cell to cell contact mediated effect or a soluble mediated effect which required direct stimulation of the cell to upregulate the required receptor. Further investigation of the B7 response will therefore be required.

It became evident that the responses seen when using the SEA blasts changed significantly depending upon the period elapsed since their last antigenic rechallenge. Up to day 4 after restimulation the blasts were proliferating maximally and further stimulation had little effect. During days 4-10 the blasts could be induced to proliferate to a single stimulus such as αCD3, PMA and B7 alone but basal proliferation remained high until day 7. After day 10 the blasts began to lose their ability to respond to B7 alone and by day 12 required full restimulation to maintain long term viability. These data are summarised in figure 5.5 which shows the timecourse of B7 stimulation. The fact that the blasts could proliferate to PMA and ionomycin on day 12 showed they had not lost viability at this time but that the B7 stimulation alone was no longer sufficient to drive proliferation.

These data demonstrated that, although not a exact representation of a resting T cell, the quiescent SEA blasts appeared to be a good physiological model in which to investigate T cell stimulation. Although the blasts could be activated by stimulation through the TCR alone this was enhanced in the presence of B7 costimulation. The ability of the SEA blasts to respond to B7 alone also provided an opportunity for further investigation of the CD28 signalling pathway independently of TCR signals making the model an extremely useful laboratory tool in the elucidation of T cell activation mechanisms.

5.2 TRANSCRIPTION FACTOR INDUCTION

Despite a failure to detect IL-2 output from the B7-stimulated blasts the induced proliferation implied the activation of downstream CD28 signalling pathways. Furthermore an increase in IL-2 output was observed in the DR4/B7-SEA stimulated blasts compared to the DR4-SEA stimulated blasts therefore a pertinent question seemed to be whether this involved an effect on transcription factor induction. Gel shift assays were therefore carried out to assess the differences of these stimulations. Unlike the problems encountered when using the PMA/ ionomycin blasts, however, it was found to be possible to quiesce the SEA-stimulated T cell blasts after antigenic challenge. Therefore whilst transcription factor analysis from unstimulated cells at day 4 revealed high levels of all three of the transcription factors studied which were not visibly altered by stimulation of the cells, when retested at day 8, basal levels were found to be very low and full restimulation of the cells using PMA and ionomycin resulted in a strong observable induction (figure 5.6).

Day 10 blasts were chosen to ensure maximum cell numbers and low basal activity. These were stimulated with fixed DR4 and DR4/B7 transfectants pulsed with SEA or B7 transfectants alone and nuclear extracts prepared. Proliferation and IL-2 assays were carried out in parallel to assess the effectiveness of the stimulations. The outcome of this assay is shown in figure 5.7. As expected the CHO control stimulation of the cells had no effect, resulting in basal expression levels of the three transcription factors, however, both DR4-SEA and DR4/B7-SEA stimulations caused maximal induction. This indicated that superantigen stimulation was capable of providing sufficient stimulation through the TCR in the absence of any costimulation. Interestingly B7 stimulation alone resulted in the generation of AP-1 and NFκB but did not induce NFAT. A comparison of these results with those obtained from the proliferation and IL-2 studies revealed an interesting correlation. Basal proliferation as observed in the CHO stimulated cells was very low whilst that induced by DR4-SEA and DR4/B7-SEA stimulation was maximal, the presence of B7 in the latter appearing to have only a minimal effect. The proliferation observed following B7 stimulation alone, although above basal levels, was fairly insignificant compared to TCR stimulation. This was in agreement with initial proliferation studies which indicated that by day 10 the ability of B7 to induce a response was downregulated, however it could clearly still induce transcription factor generation and some proliferation (figure 5.8A). Results from the IL-2 assay revealed that whilst IL-2 was produced by both DR4-SEA and DR4/B7-SEA stimulations the output from the latter was considerably greater indicating a costimulatory role for B7 in IL-2 generation. Furthermore the absence of IL-2 production in CHO and B7 stimulated cells correlated with an absence of NFAT production. This implied that NFAT induction may play a crucial role in IL-2 gene transcription (figure 5.8B).

The absence of NFAT induction indicated that the B7 stimulation was specifically activating the CD28 costimulatory pathway and not delivering a global stimulation as was seen with PMA and ionomycin and further supports the finding that normal T cells require two signals for full activation. The identification of AP-1 and NFκB as two downstream targets of CD28 activation which could be induced in the absence of concomitant TCR ligation provided a useful end point to investigate the signalling pathways associated with CD28. Further investigation revealed that inducibility of factors by B7 no longer occurred after day 12 post antigenic challenge. All experiments were therefore carried out on blasts between day 8 and 10 post antigenic challenge.

5.3 INVESTIGATION OF CD28 SIGNALLING PATHWAYS

Since CD28 signalling has been reported to involve the enzymes PI3 Kinase and acidic sphingomyelinase, inhibitors of these enzymes were investigated. Wortmannin was used

to inhibit PI3 Kinase activity whilst the lysosomotropic, alkalating agent chloroquine was used to inhibit acidic sphingomyelinase activation.

Wortmannin is a fungal metabolite which acts by irreversibly binding and hence blocking the p110 catalytic subunit of PI3 Kinase. However, previous studies have shown that in the presence of wortmannin PI3 Kinase association with CD28 is still observed. At high concentrations (> 1μM) wortmannin has been found to inhibit other enzymes but below this concentration it is thought to be specific for PI3 Kinase. Its action is very rapid and will induce a complete block within 15 minutes however, the half life of the compound in aqueous solution is extremely short (< 4 hours). Since the enzyme inhibition is irreversible, overcoming wortmannin treatment requires new enzyme synthesis, the rate of which appears to parallel the proliferation rate of the cell. Figure 5.9 shows the effect of a wortmannin titration on the proliferation of resting T cells costimulated with αCD3 and CHO-B7 transfectants. Although the inhibition was not absolute at doses of 100nM it was clear that the extent of the inhibition of proliferation was dose dependent. When repeated using the SEA blasts and Jurkat cells, however, it was found that wortmannin had very little effect on proliferation (figure 5.10). Slight inhibition was noted in the basal proliferation of the quiescent blasts which increased to an approximate 25% inhibition in the B7 stimulated cells. This may indicate some involvement of PI3 Kinase but it does not appear to be essential. Wortmannin had even less effect on Jurkat proliferation but appeared to affect the basal proliferation rate to a slightly greater extent than the B7 stimulated cells.

Since JNK and hence AP-1 activation are proposed to be associated downstream signalling events of PI3 Kinase its activation was implicated in the induction of AP-1 by B7. The failure of wortmannin to inhibit B7 induced proliferation in the SEA blasts therefore made the role of PI3 Kinase in these T cells difficult to assess. A biochemical assay was carried out to investigate the regulation of JNK activation in the T cell blasts and to test its sensitivity to inhibition by wortmannin. Figure 5.11A shows evidence of enzyme activity in Jurkat cells activated by U.V. light whilst figure 5.11B shows the phosphorylated GST-jun product in B7 stimulated T cell blasts.

Since CD28 signalling has also been proposed to involve the activation of acidic sphingomyelinase, the role of this enzyme was also investigated. Low dose chloroquine was extremely effective at inhibiting the B7 induced proliferative response (figure 5.12) although higher doses were found to be cytotoxic over long periods of time (>24 hours). The ability of B7 stimulation to directly activate acidic sphingomyelinase in the SEA T cell blasts was also investigated using a biochemical assay to measure enzymatic activity. Figure 5.13 shows the percentage increase in enzyme activity above basal levels (around 700cpm) following B7 stimulation over time as measured by the generation of ^{14}C radiolabelled phosphorylcholine

a substrate of the hydrolysis reaction and which was inhibited by preincubation of the cells with chloroquine. This did not occur in SEA T cell blasts stimulated with αCD3 indicating it was a specific response involving activation of the costimulatory pathway. However, since DR4-SEA stimulation seemed to be capable of activating the T cell blasts the effect of this stimulus on enzymatic activity was also investigated. No induction was observed using DR4 cells in the absence of antigen but did occur when cells were stimulated with DR4-SEA or DR4/B7-SEA (figure 5.14). These results indicate that SEA and αCD3 do not provide comparative signals and that the SEA may also activate at least some aspects of the costimulatory signalling pathway.

Having established that B7 stimulation of the T cell blasts did indeed activate sphingomyelinase and may have been activating downstream targets of PI3 Kinase and and that these pathways could be blocked effectively with chloroquine and wortmannin respectively the next step was to assess the effect of these inhibitors directly on the induction of the AP-1 and NFκB transcription factors. Gel shift assays were performed using nuclear extracts prepared from cells incubated with the relevant inhibitor prior to B7 stimulation. Figures 5.15(A) and (B) show the outcome of these assays. Whilst NFκB induction was unaffected by wortmannin it was completely inhibited by preincubation of the cells with chloroquine. In contrast AP-1 induction was partially inhibited by wortmannin but demonstrated variable sensitivity to chloroquine. Whilst no effect was seen with chloroquine in some assays (B(i)) others resulted in partial or complete inhibition of AP-1 (B(ii)). The present data therefore indicate that whilst PI3 Kinase may be involved in the induction of AP-1, sphingomyelinase appeared to regulate both AP-1 and NFκB.

As wortmannin did not appear to have a significant effect on the B7-induced proliferation in T cell blasts and activation of the sphingomyelinase pathway alone is reported to induce apoptosis, the involvement of additional " survival" signals in the generation of proliferation and IL-2 production was implicated. Other signalling molecules which may have been involved in the B7 induced activation and which were investigated were S6 kinase, PKC enzymes and calcium (figure 5.16). Although S6 kinase has been proposed as a possible downstream target of PI3 kinase, unlike wortmannin, rapamycin, a known inhibitor of S6 kinase activation, did produce a dramatic inhibition of B7-induced proliferation in the T cell blasts. However, since it also induced an inhibition of basal proliferation this may be related to an inhibition of cell cycle progression. The involvement of PKC enzymes were investigated by preincubation of the cells with the PKC inhibitor Ro 31-8220 which slightly reduced the basal proliferation levels in the T cell blasts but significantly inhibited the B7 stimulated response. This indicated that either a PKC enzyme is a downstream target of CD28 signalling or that a PKC-derived signal is required to synergise with the CD28 signals to

produce the observed B7 response. Finally, despite CD28 signal transduction being reported as calcium independent, inclusion of CsA, which blocks calcineurin activation by the calcium / calmodulin complex, did inhibit the B7-induced proliferation in blasts whilst having negligible effects on basal turnover. Furthermore an interesting observation was noted when the effects of CsA were examined on B7-costimulated PMA/ionomycin-treated SEA blasts. Both the PMA/ionomycin induced proliferation and IL-2 output were found to be completely abrogated following CsA treatment as would be expected due to the inhibition of the ionomycin-mediated calcium signal . However, whilst the presence of CHO cells had no effect on these results, the CsA inhibition was substantially reversed in the presence of B7 transfectants (figure 5.17(A)). This indicated that the synergy of the PMA and B7 signals could overcome or bypass the CsA block. Similar responses were observed for IL-2 output although the B7-induced recovery was less complete.

Unfortunately there was insufficient time to assess the relative contributions of these other signalling pathways, as inhibited by rapamycin, Ro 31-8220 and cyclosporin A, on the B7 mediated transcription factor induction and which will therefore require further study in the future.

5.4 SUMMARY

Using the B7 responsive SEA blasts as a model of resting T cells has provided some significant new information on the role of CD28 in T cell activation and the signalling pathways it employs to undertake this role. The response to B7 alone was shown to be time dependent indicating a requirement of some prior activation of the cell but since no NFAT complexes or IL-2 production were observed this appeared to discount a fully activated TCR pathway. Furthermore although CD28 signalling is reported to be calcium independent the B7 response was inhibited by cyclosporin A and yet B7 stimulation in conjunction with PMA and ionomycin could significantly overcome the cyclosporin A block of calcium signalling to induce IL-2 production. Finally CD28 activation was shown to induce the AP-1 and NFκB transcription factor complexes via activation of the PI3 Kinase and acidic sphingomyelinase signalling pathways although further investigation will be required to discover the exact mechanisms and control points of these pathways.

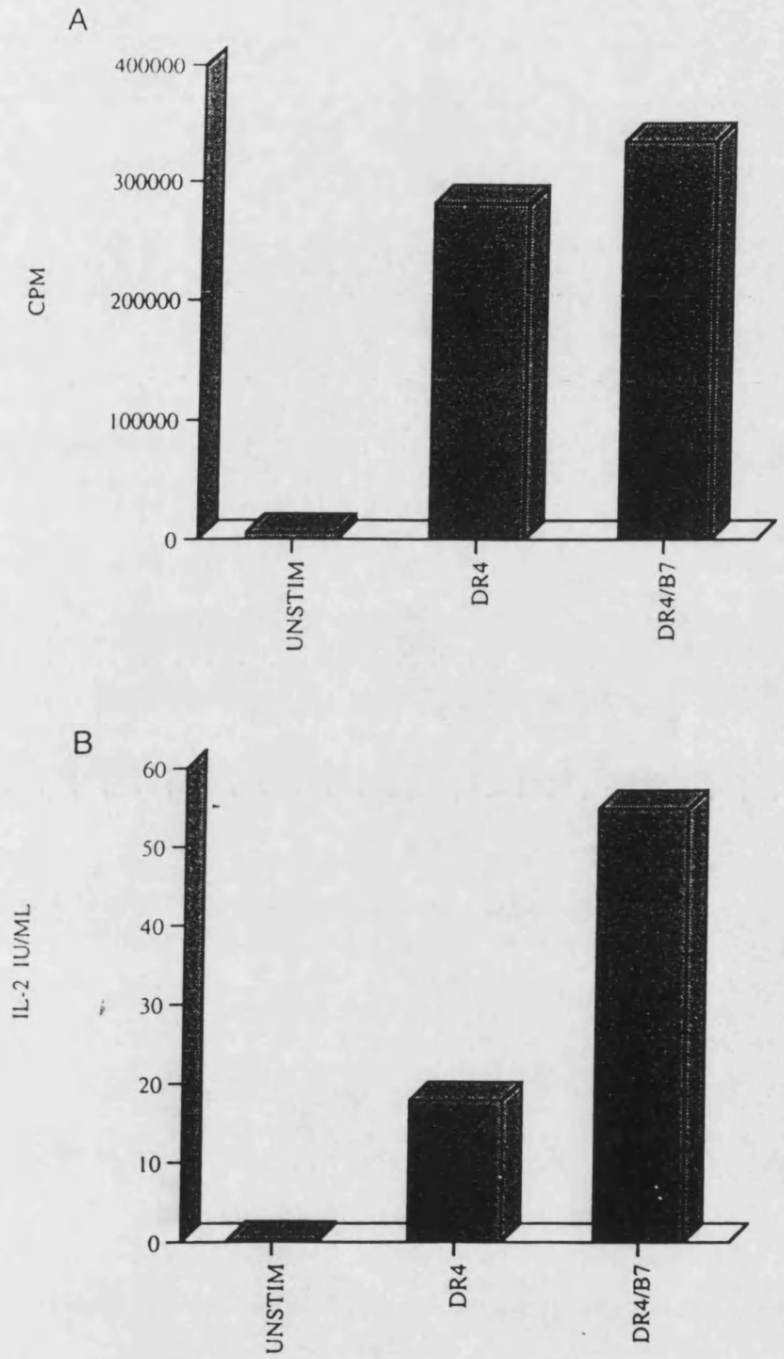


Figure 5.0 Effect of costimulation on superantigen induced activation of T cell blasts

Day 8 SEA T cell blasts were left unstimulated or were incubated with CHO-DR4 transfectants pulsed with SEA (10ng/ml) alone or in the presence of cotransfected B7 for 72 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

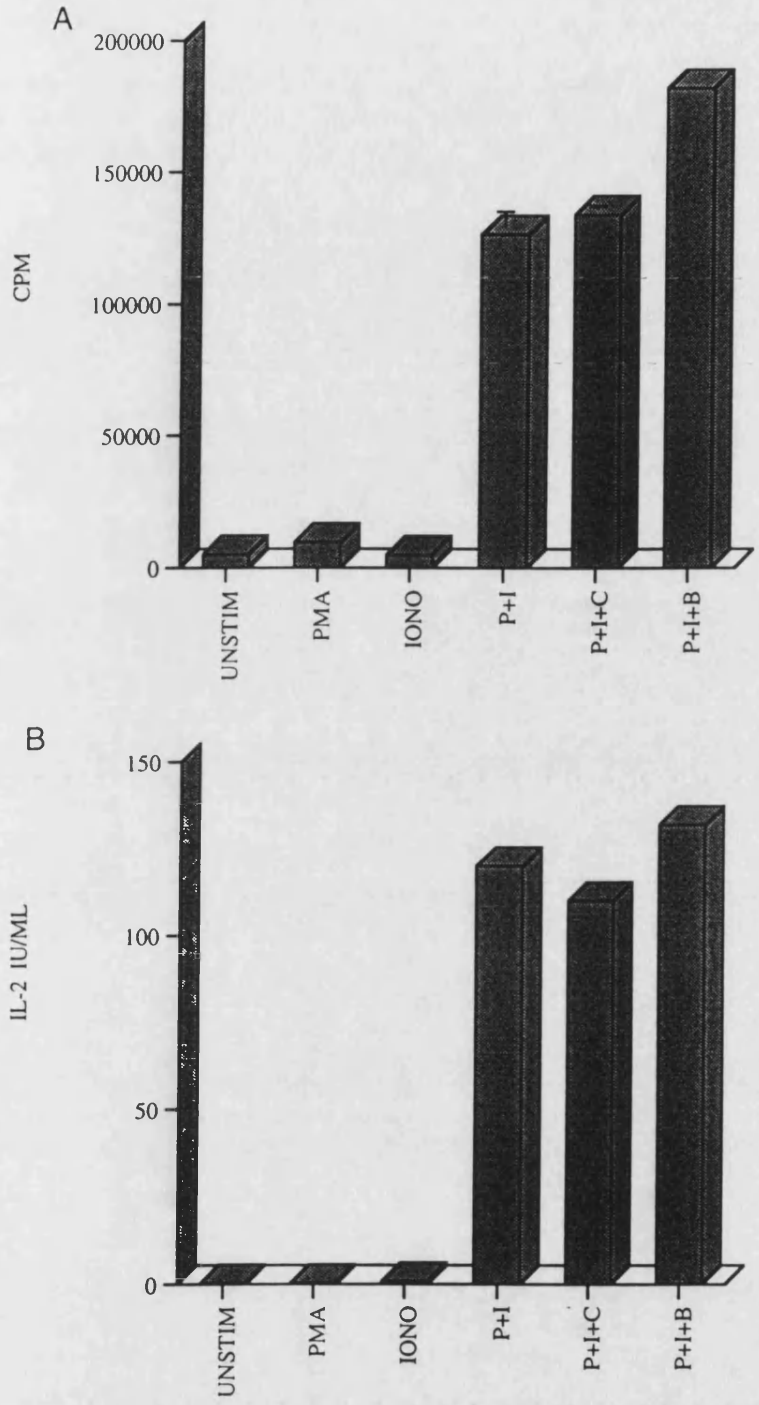


Figure 5.1 Effect of costimulation on mitogen induced activation of T cell blasts

Day 8 SEA T cell blasts were left unstimulated or were incubated with PMA (0.3ng/ml) and ionomycin (1μM) alone or in the presence of fixed CHO cells or fixed B7 cells for 72 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

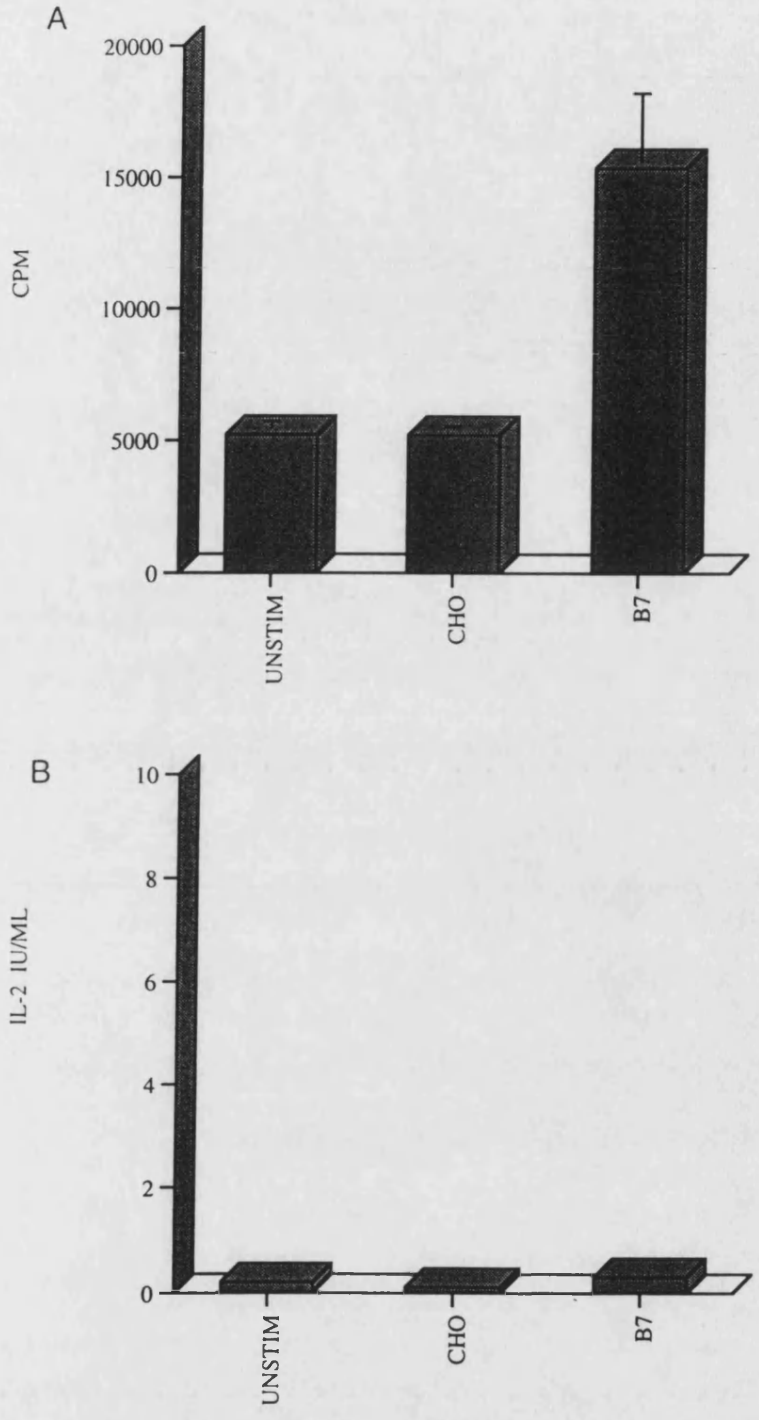


Figure 5.2 Effect of CHO-B7 on the proliferation and IL-2 production in T cell blasts

Day 8 SEA T cell blasts were left unstimulated or were incubated with fixed CHO cells or fixed B7 cells for 72 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

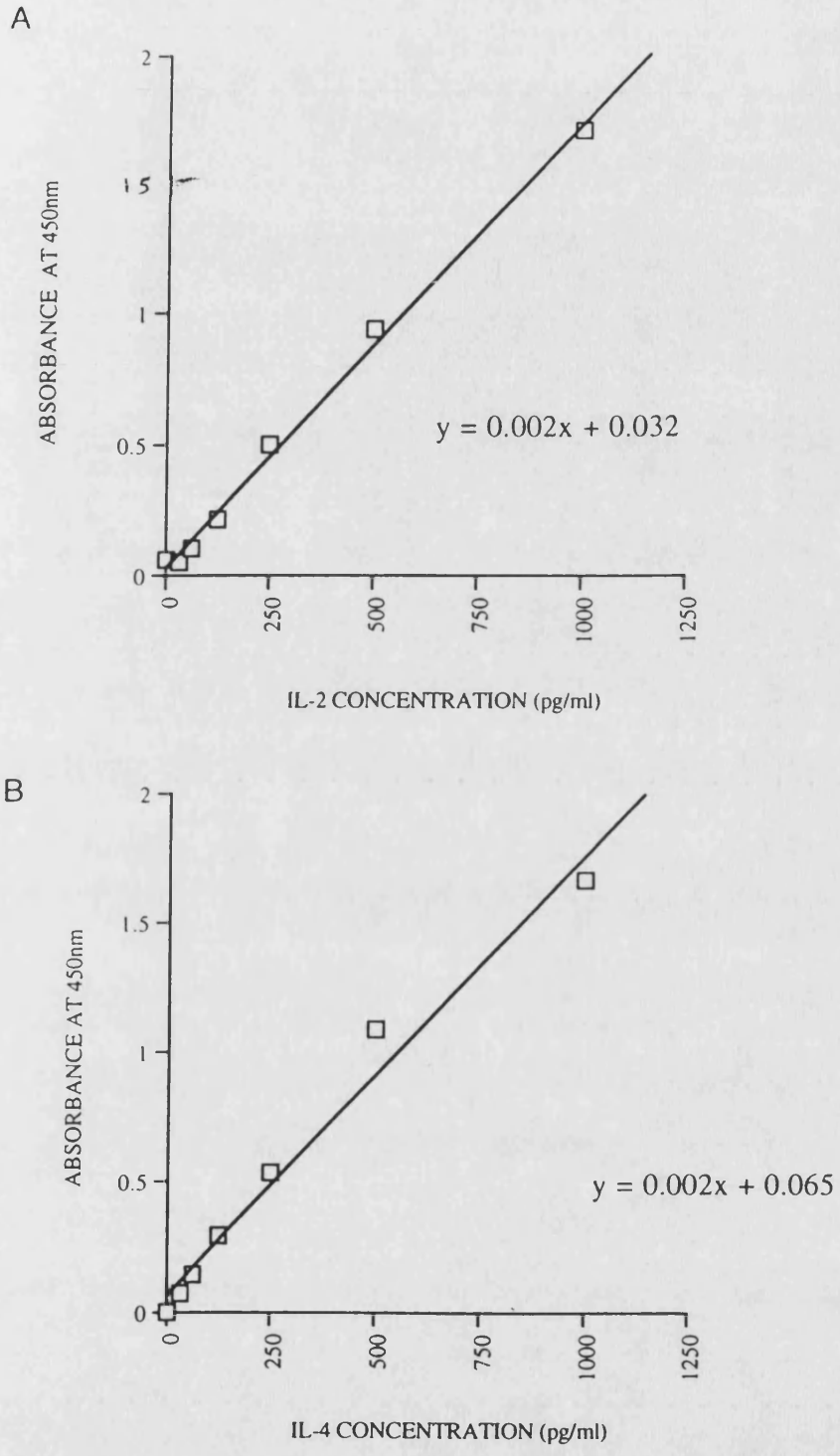


Figure 5.3 Cytokine standard curves for ELISA

ELISA calibration curves were prepared by serial dilution of recombinant IL-2 (A) and IL-4 (B) standards. Samples were diluted in the range (0.1-0.001) and were analysed by spectrophotometry at 450nm.

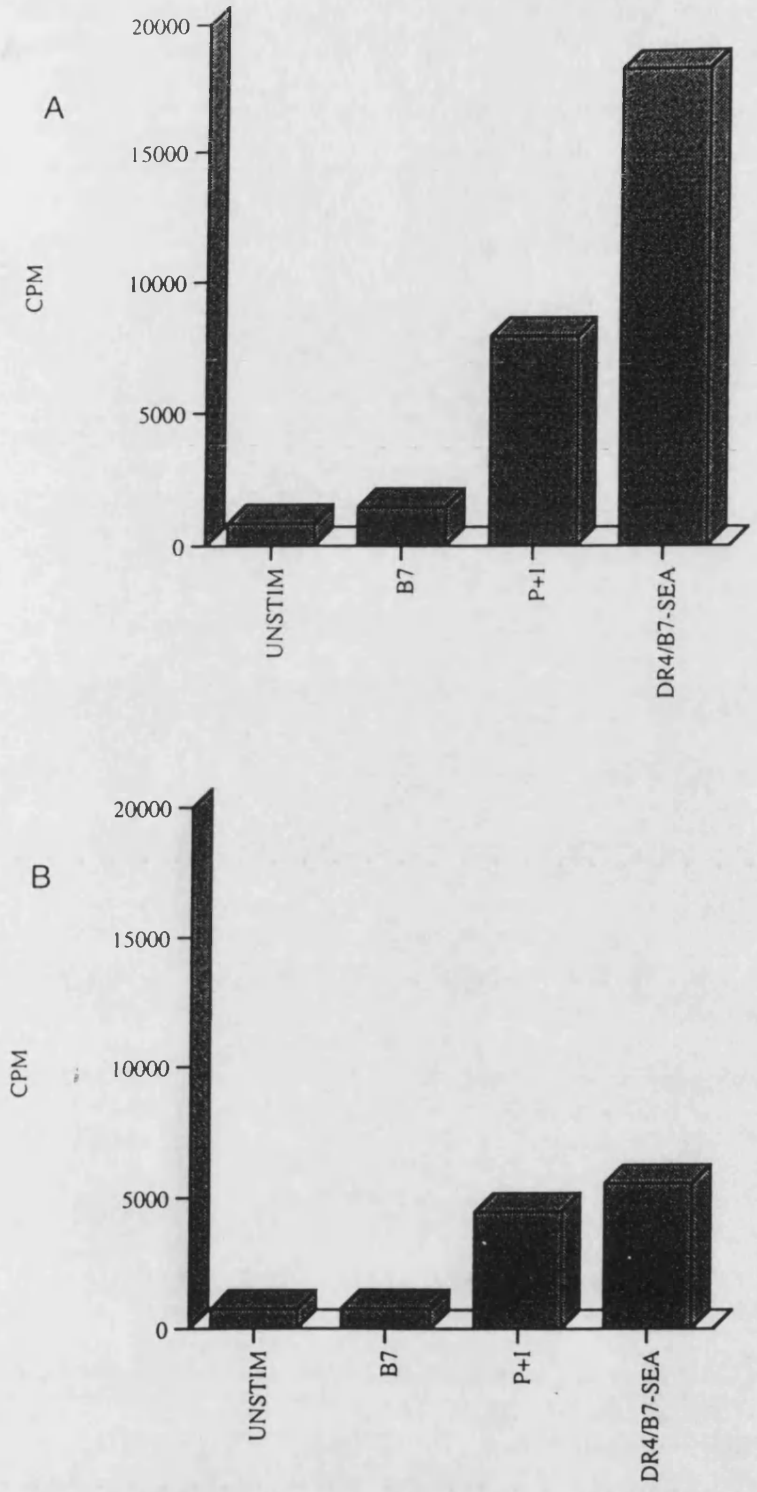


Figure 5.4 Induction of proliferation in SEA blasts by supernatant transfer

Day 8 SEA blasts were incubated in a 96 well plate with media (UNSTIM), CHO-B7 transfectants (B7), PMA (0.3ng/ml) and ionomycin (1μM) (P+I) or DR4/B7-SEA for 24 hours (A). Aliquots of supernatant werethen removed from these wells and added to fresh unstimulated blasts (B). Labels represent origin of supernatant. After a further 48 hours the proliferation of both sets of blasts were analysed by ^{3}H-Thymidine incorporation. Data are the triplicate mean of a single experiment.

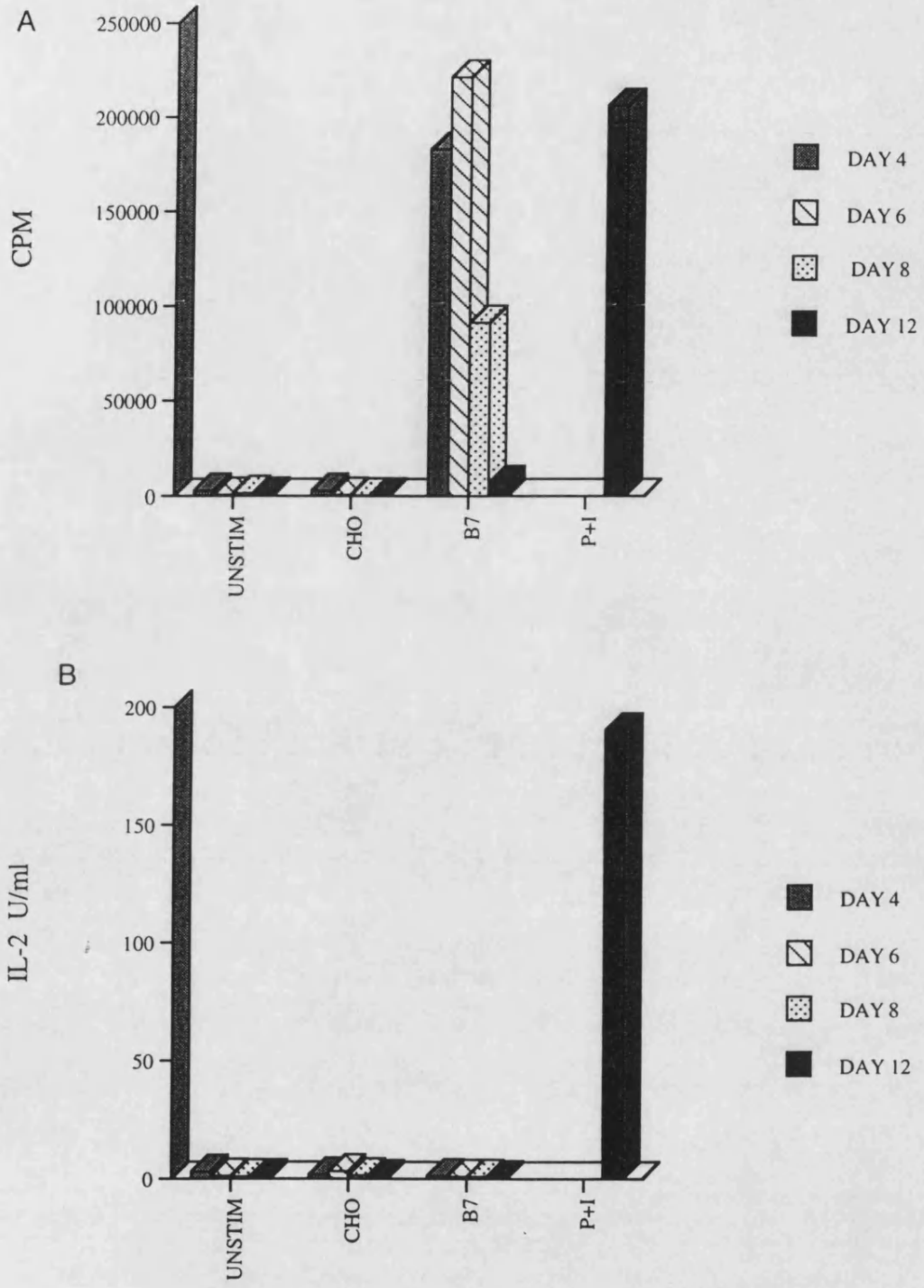


Figure 5.5 A timecourse of B7-induced stimulation of T cell blasts

SEA T cell blasts, taken from culture at the specified day after the last restimulation and were left unstimulated or were incubated with fixed CHO cells , fixed B7 cells or PMA(0.3ng/ml) and ionomycin (1μM) for 24 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

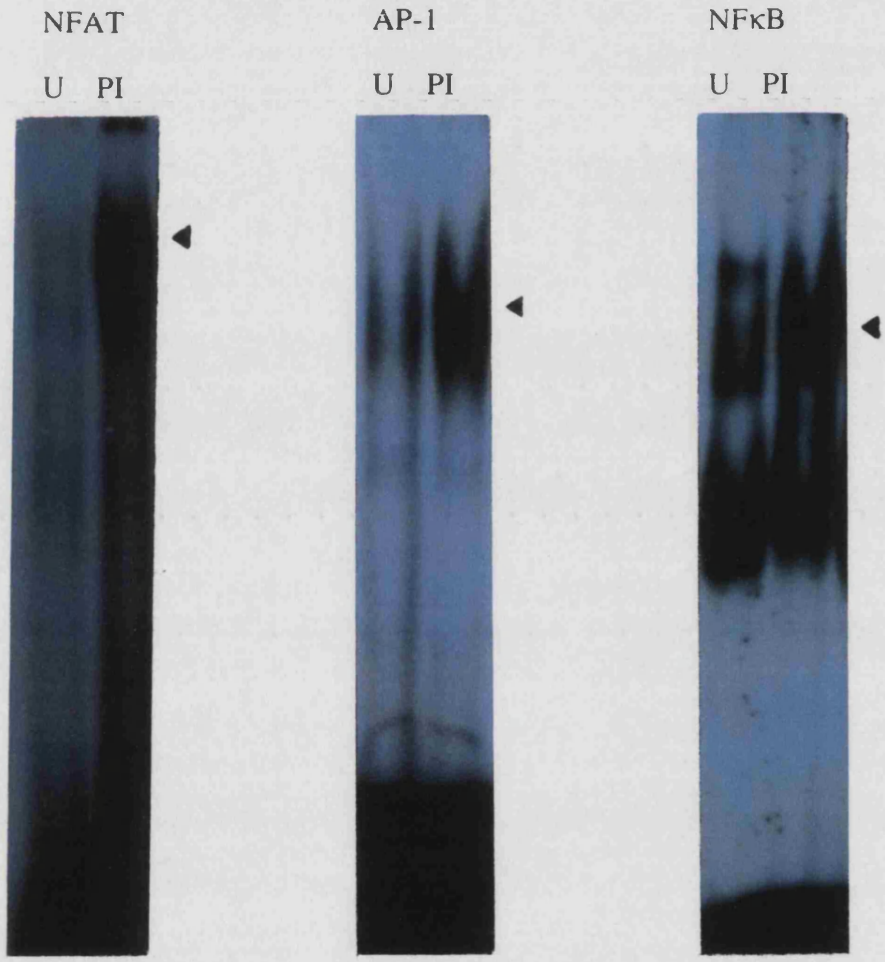


Figure 5.6 Induction of transcription factor complexes.

Nuclear extracts were prepared from unstimulated (U) and PMA (0.3ng/ml) / ionomycin (1μM) stimulated Day 8 SEA T cell blasts and were analysed for the production of NFAT, AP-1 and NFκB complexes.These were visualised by autoradiography following an overnight exposure of the gel.

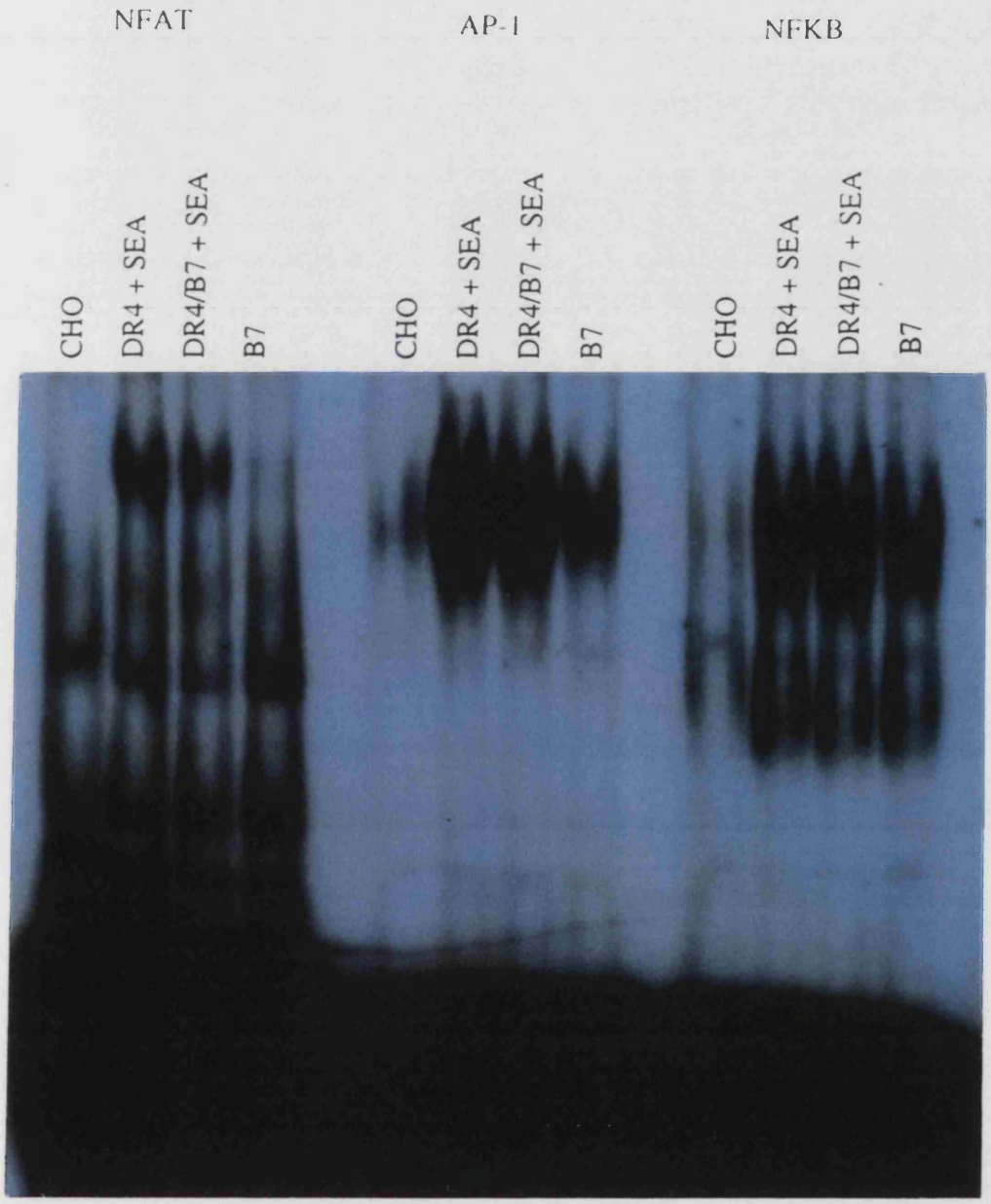


Figure 5.7 Induction of transcription factor complexes.

Nuclear extracts were prepared from CHO, DR4-SEA, DR4/B7-SEA and B7 stimulated Day 10 SEA T cell blasts and were analysed for the production of NFAT, AP-1 and NFκB complexes.These were visualised by autoradiography following an overnight exposure of the gel.

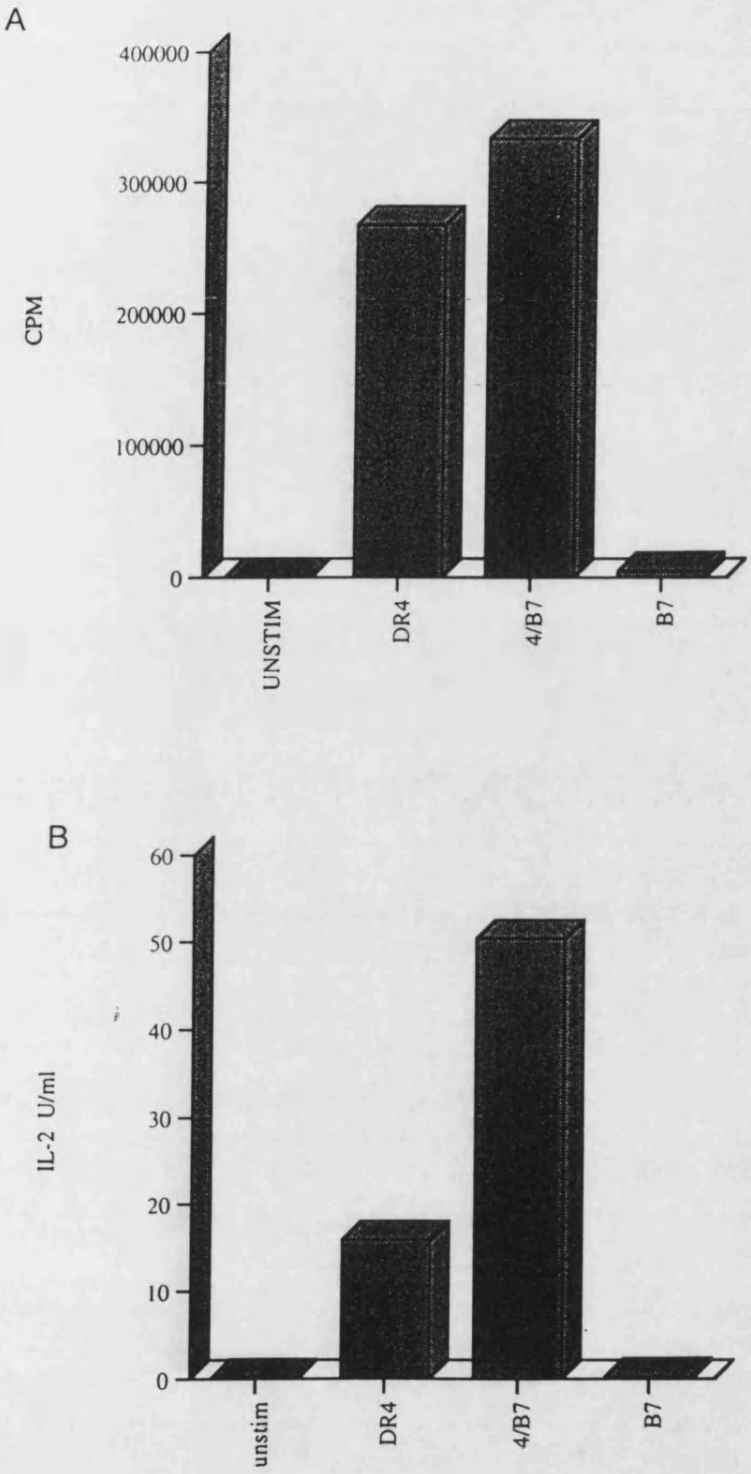


Figure 5.8 Proliferation and IL-2 production in stimulated T cell blasts

Aliquots of Day 8 SEA T cell blasts were taken from the incubated cultures of cells prior to the preparation of nuclear extracts and the cells were then incubated for a further 24 hours. Proliferation (A) of these aliquots was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

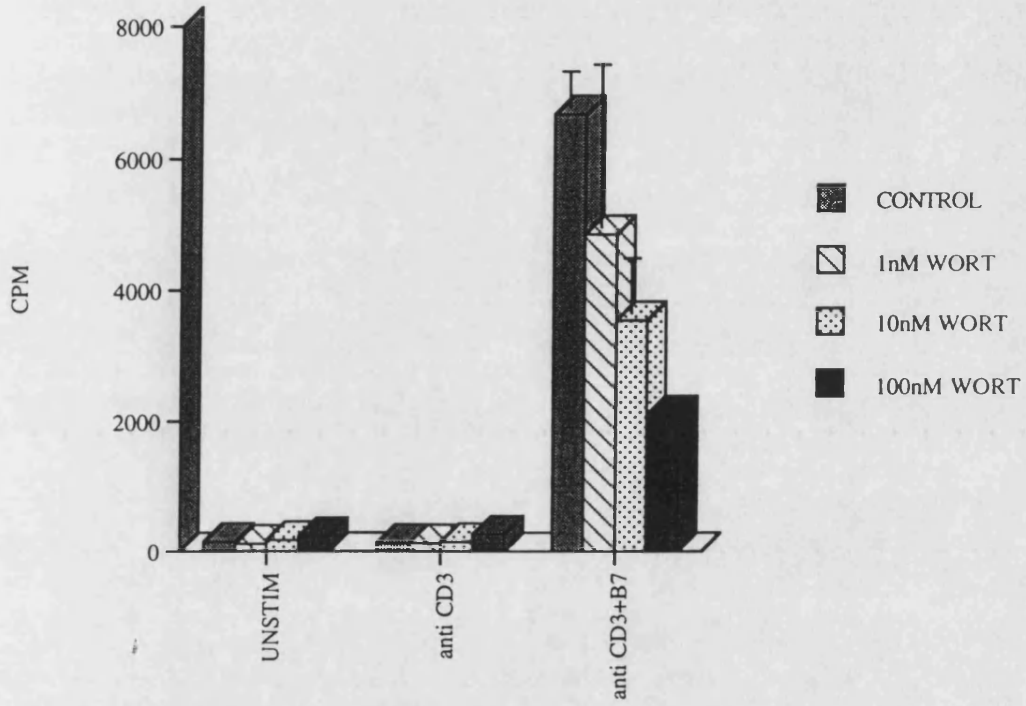


Figure 5.9 Effect of Wortmannin on the proliferation of resting T cells

Resting T cells were preincubated for 30 minutes with media or wortmannin (100nM). The cells were then placed in media or were incubated with αCD3 (1μg/ml) alone or in the presence of fixed B7 cells for 24 hours. Proliferation was measured by ^{3}H-Thymidine incorporation and data are the triplicate mean of a representative experiment.

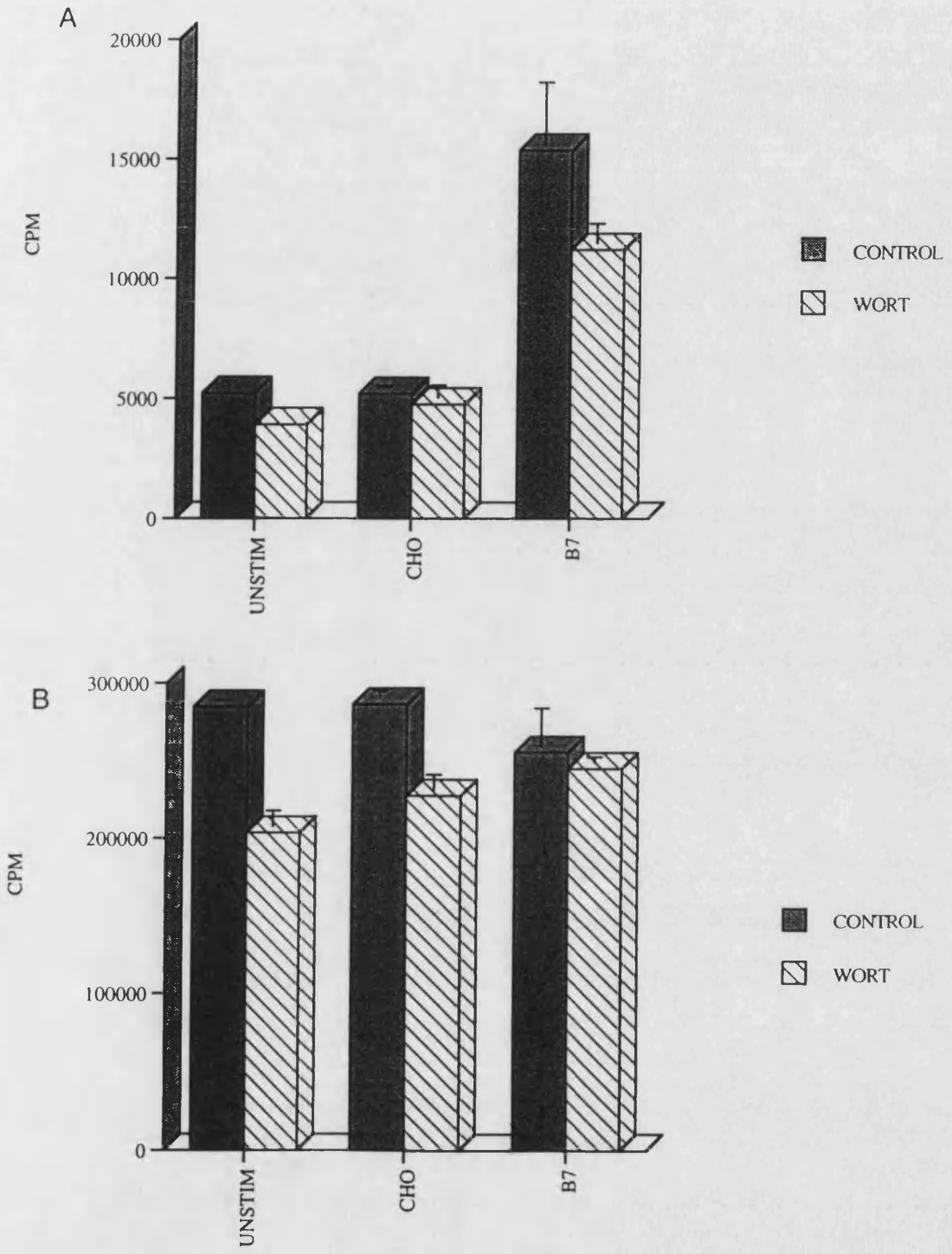


Figure 5.10 Effect of Wortmannin on the proliferation of T cell blasts and Jurkat cells

Day 8 SEA T cell blasts (A) and Jurkat cells (B) were preincubated for 30 minutes with media or wortmannin (100nM) and were then placed in media alone or were incubated with fixed B7 cells for 24 hours. Proliferation was measured by ^{3}H-Thymidine incorporation and data are the triplicate mean of a representative experiment.

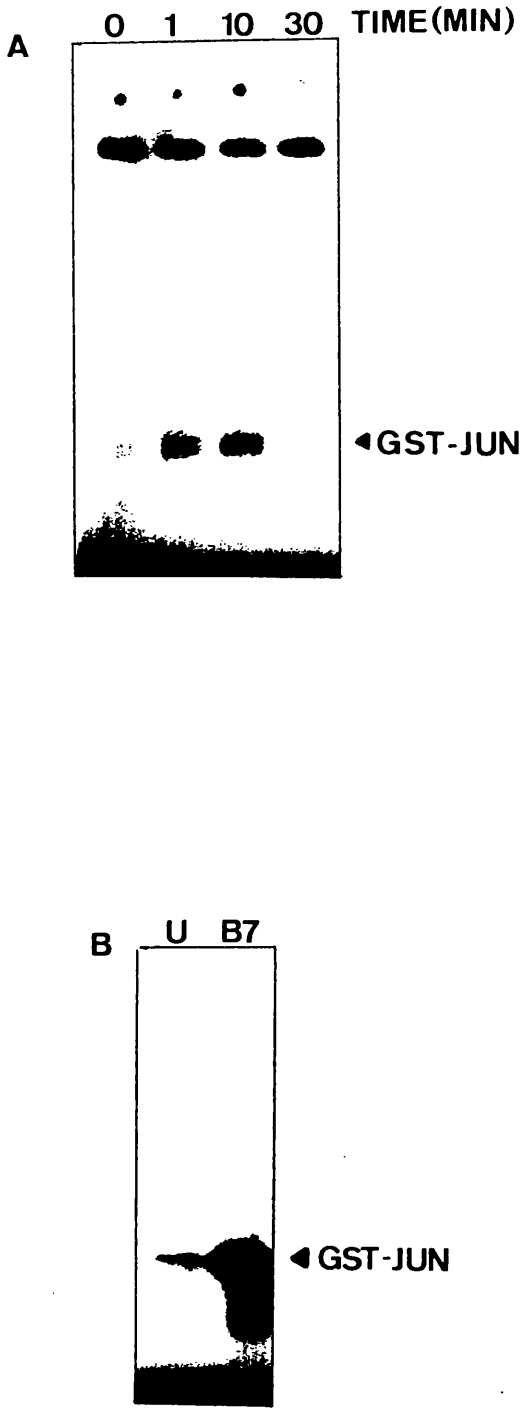


Figure 5.11 Induction of JNK activity

Jurkat cells were exposed to U.V. light for the time specified (A) whilst Day 8 SEA blasts were left unstimulated or were incubated with CHO-B7 transfectants for 1 hour (B) Cytoplasmic extracts were then prepared and assayed for enzymatic activity.

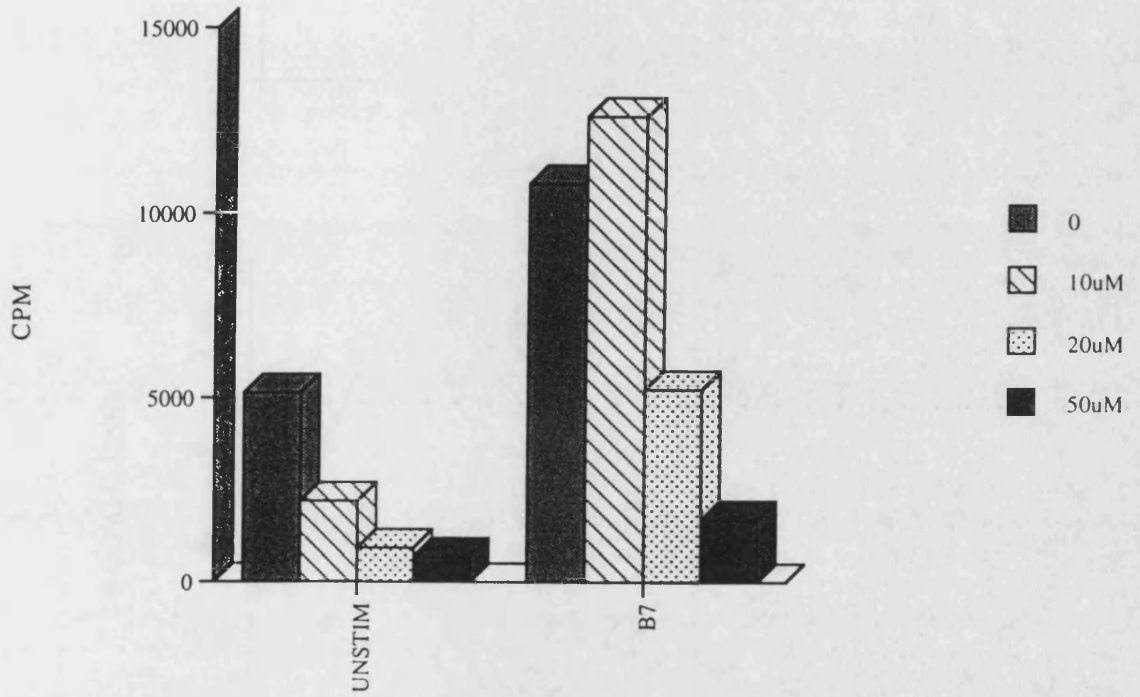


Figure 5.12 Effect of Chloroquine on the B7-induced proliferation in T cell blasts

The SEA T cell blasts were preincubated for 1 hour with media or chloroquine at the concentrations specified. The cells were then placed in media alone or were incubated with fixed B7 cells for 24 hours. Proliferation was measured by ^{3}H-Thymidine incorporation and data are the triplicate mean of a representative experiment.

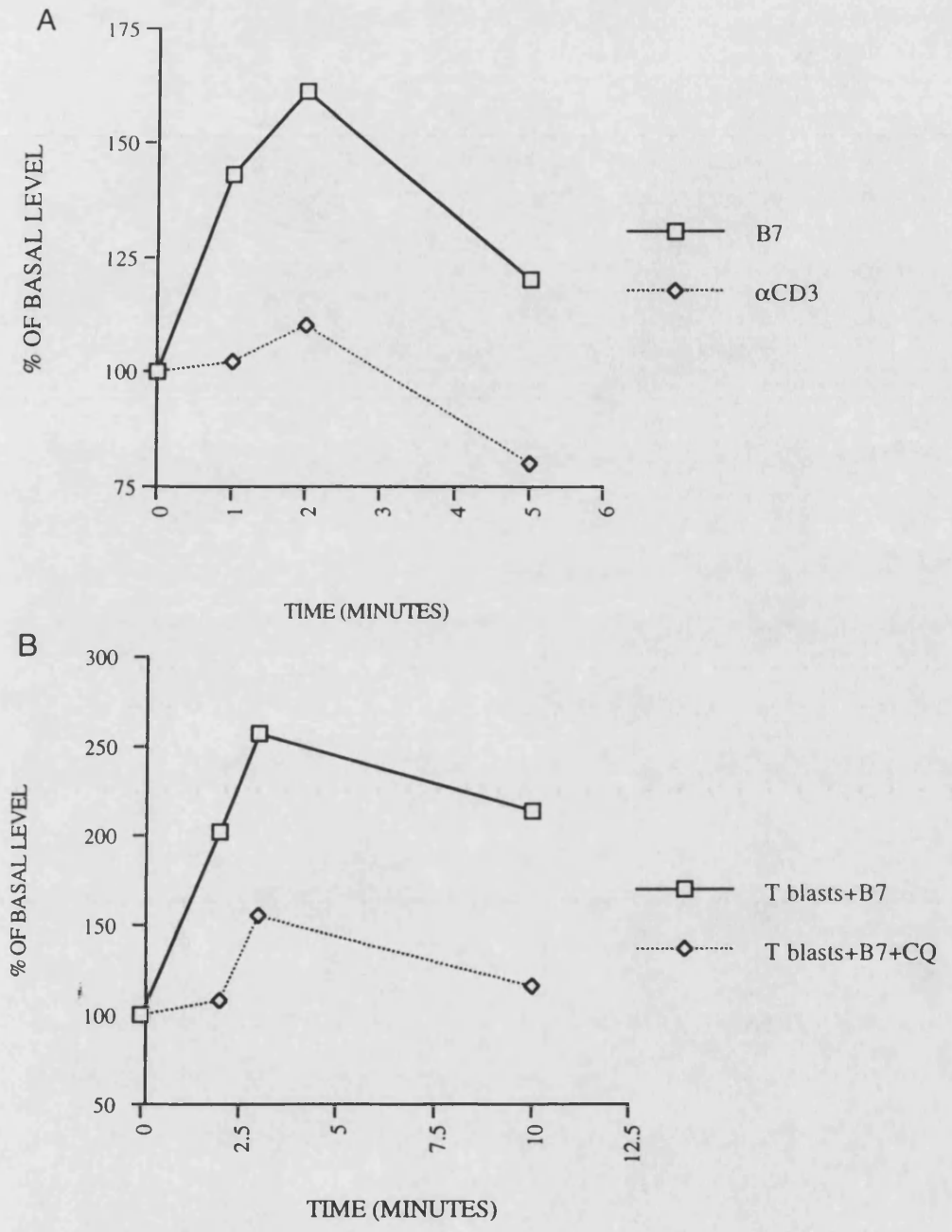


Figure 5.13 Induction of sphingomyelinase activity in B7 and αCD3 stimulated SEA T cell blasts

SEA T cell blasts were preincubated for 1 hour in serum free media alone or in the presence of chloroquine (200μM) prior to incubation with CHO-B7 cells (A) or αCD3 (1μg/ml)(B) for the time specified. The cells were then washed and cytoplasmic extracts were prepared. Enzymatic activity in the extracts was assessed using an *in vitro* biochemical assay as detailed in the methods section and measured by β liquid scintillation counting. The graph shows percentage enzyme activity as a factor of stimulation time in the presence an absence of chloroquine. Basal counts were typically around 700cpm. Data are from a representative experiment.

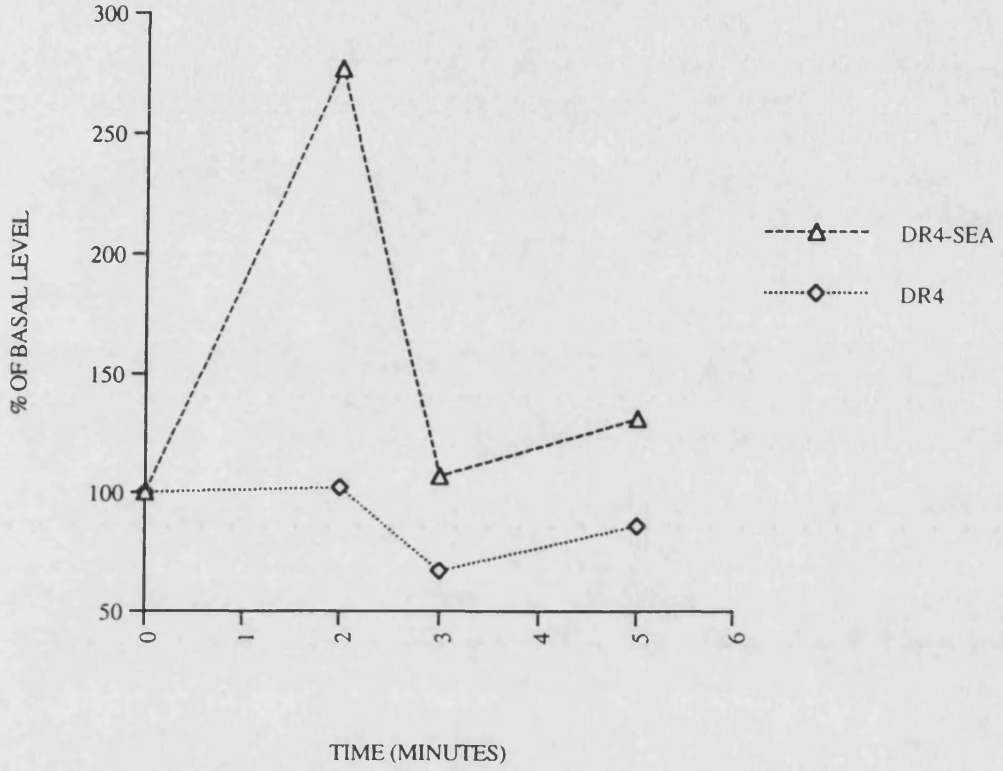


Figure 5.14 Induction of sphingomyelinase activity in DR4 / DR4-SEA stimulated T cell blasts

SEA T cell blasts were preincubated for 1 hour in serum free media alone or in the presence of chloroquine (200μM) prior to incubation with fixed DR4 or fixed DR4-SEA transfectant cells for the time specified. The cells were then washed and cytoplasmic extracts were prepared. Enzymatic activity in the extracts was assessed using an *in vitro* biochemical assay as detailed in the methods section and measured by β liquid scintillation counting. The graph shows enzyme activity as a factor of stimulation time in the presence an absence of chloroquine. Data is from a representative experiment.

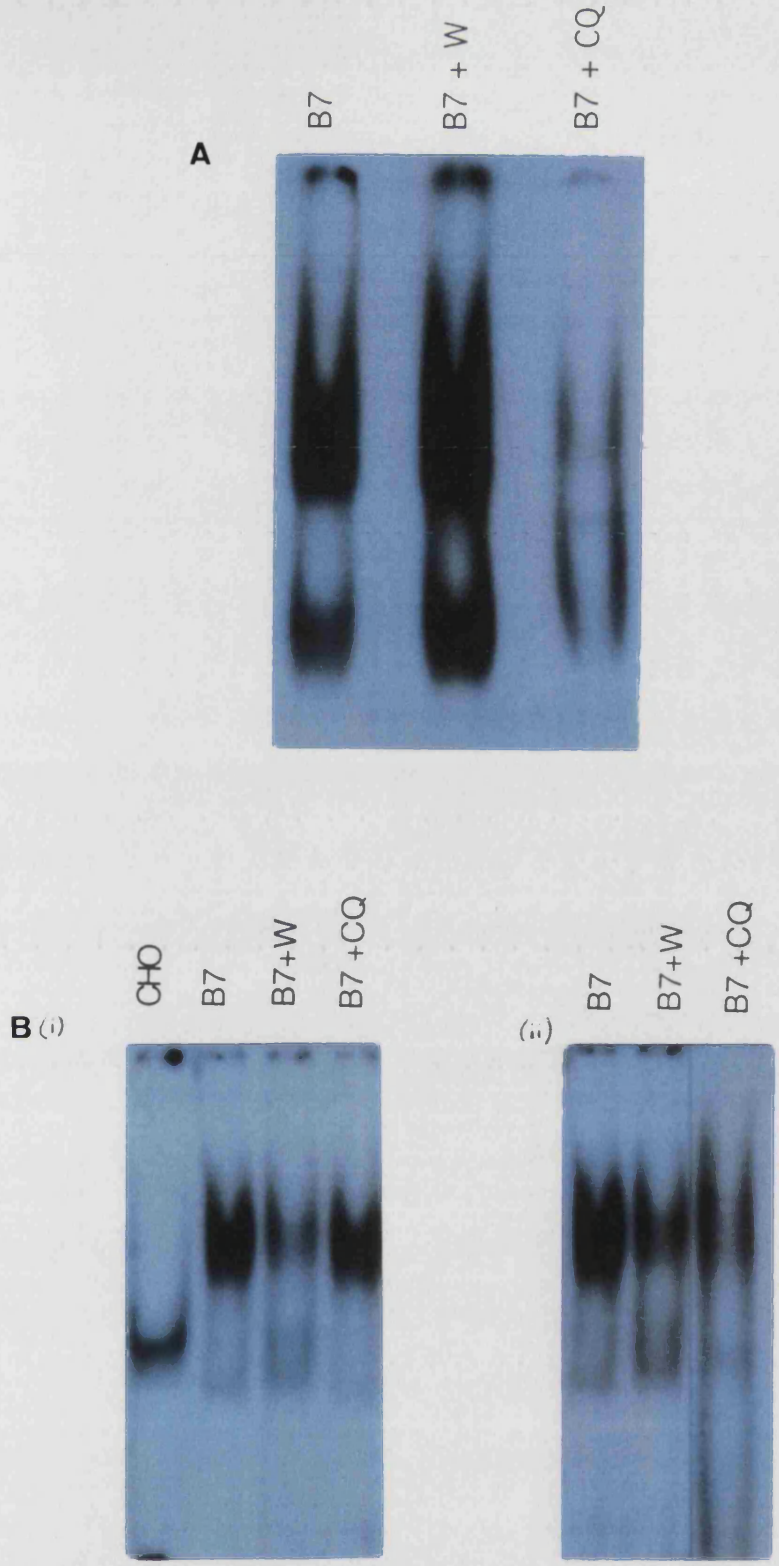


Figure 5.15 Effect of wortmannin and chloroquine on B7 generated transcription factor induction.

Day 8 SEA T cell blasts were preincubated for one hour in media (B7), wortmannin (100nM) (B7+W) or chloroquine (50μM) (B7+CQ) prior to a four hour stimulation with B7 transfectants. Nuclear extracts were prepared and analysed for NFkB (A) or AP-1 (B) induction. AP-1 demonstrated a variable inhibition to chloroquine (B(i) and B(ii)). The complexes were visualised by autoradiography following an overnight exposure of the gel.

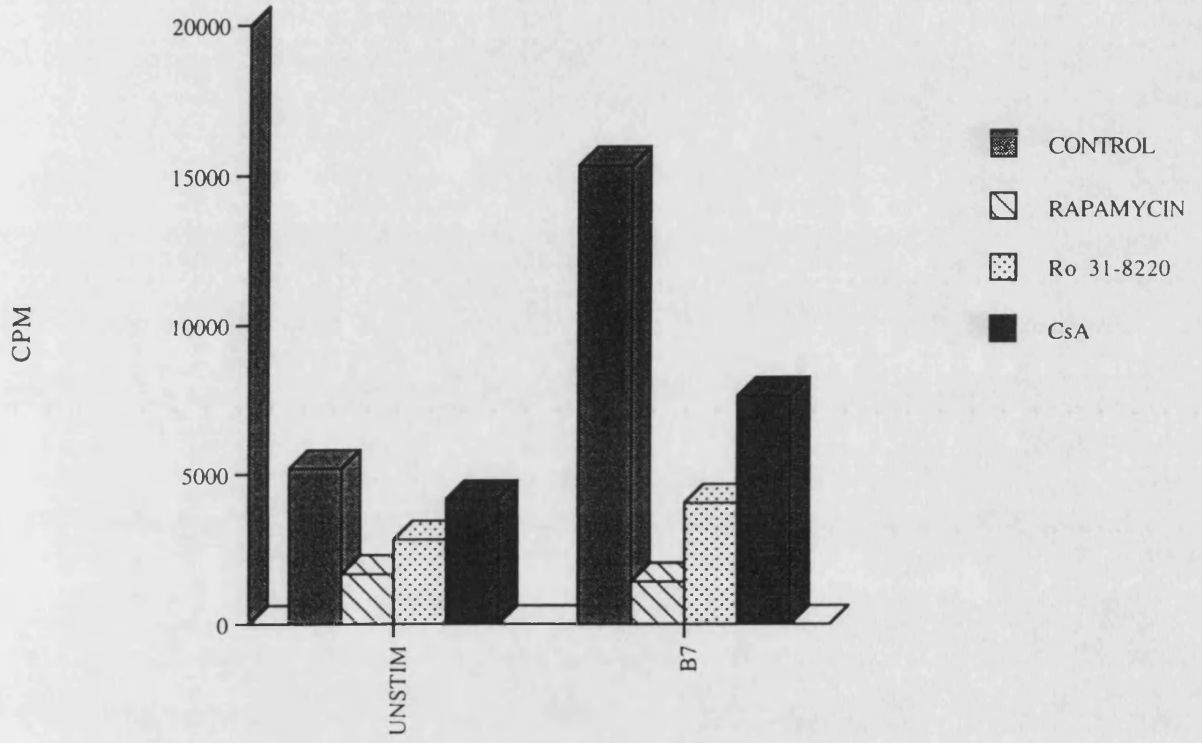


Figure 5.16 Effect of various inhibitors on the B7-induced proliferation in T cell blasts

The SEA T cell blasts were preincubated for 1 hour with media, rapamycin (10nM), Ro 31-8220 (1μM) or cyclosporin A (5μg/ml). The cells were then placed in media alone or were incubated with fixed B7 cells for 24 hours. Proliferation was measured by ^{3}H-Thymidine incorporation and data are the triplicate mean of a representative experiment.

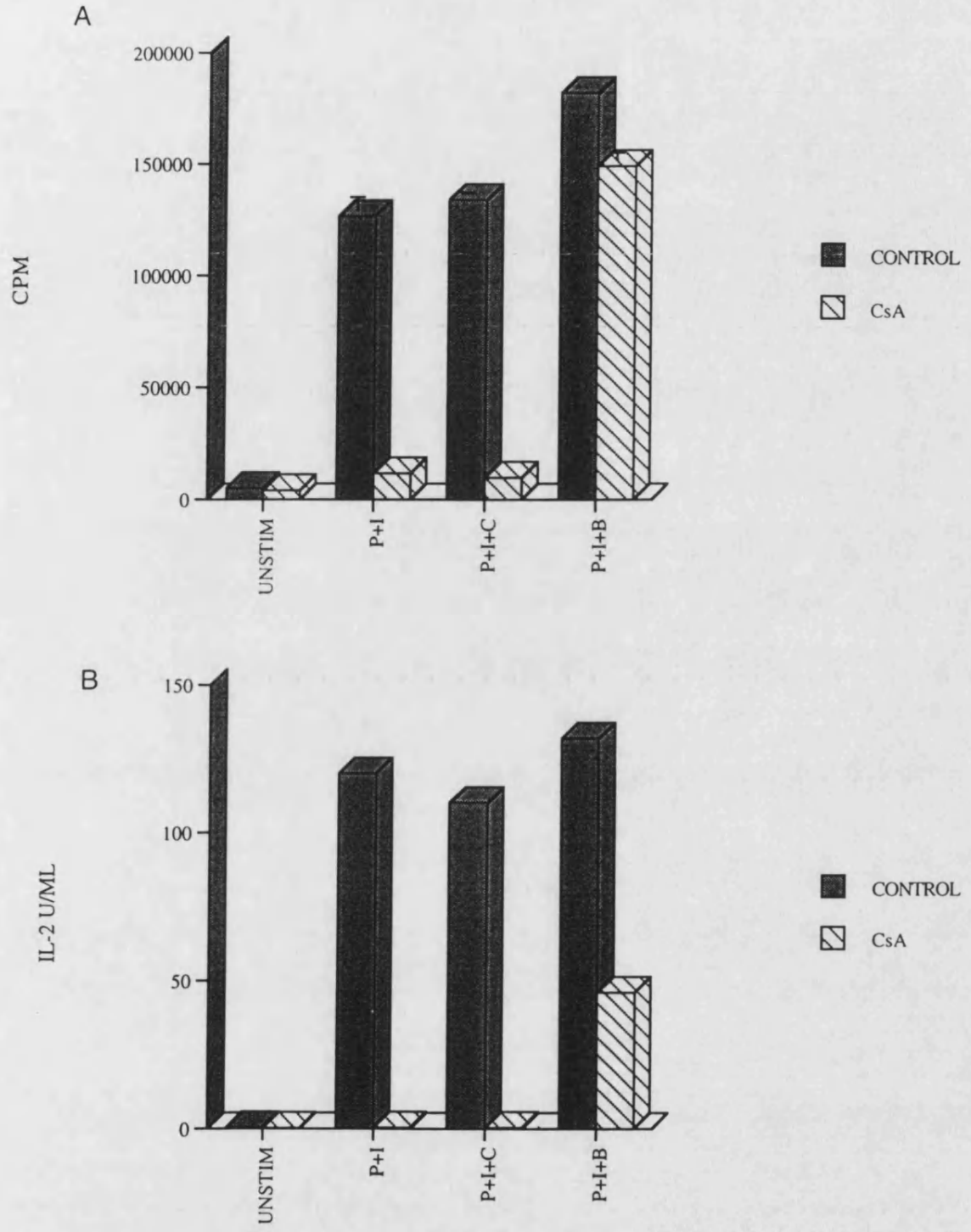


Figure 5.17 Effect of costimulation on cyclosporin A-inhibited, mitogen-stimulated T cell blasts

SEA T cell blasts were preincubated for 1 hour with media or cyclosporin A (5μg/ml). The cells were then placed in media or were incubated PMA (0.3ng/ml) and ionomycin (1μM) alone or with fixed B7 cells for 24 hours. Proliferation (A) was measured by ^{3}H-Thymidine incorporation whilst IL-2 production (B) was assessed using a CTLL bioassay. Data are the triplicate mean of a representative experiment.

5.5 DISCUSSION

Whilst the effects of costimulation on the responses of PMA/ ionomycin activated T cell blasts could be observed in proliferation and IL-2 assays it was not possible to detect any qualitative or quantitative differences in the transcription factor assays. Therefore other methods of generating T cell blasts, which did not result in such a prolonged activated phenotype, were investigated. It was found that T cell blasts induced with superantigen generated large numbers of cells which could easily be quiesced and then fully reactivated with antigenic and costimulatory signals, therefore mimicking a resting peripheral blood T cell. Although superantigen stimulation does not exhibit the same TCR restriction as observed with natural antigenic peptides, it is still dependent upon the αβ chains of the T cell receptor (Kappler et al., 1989) and therefore shows MHC dependence and antigen specificity in the activation of T cells.

The requirement for costimulation of superantigen challenged T cells has been a matter of some debate. Whilst Damle et al (Damle et al., 1993) proposed the activation was costimulation independent as indicated by the failure of a CTLA-4 Ig to inhibit superantigen driven proliferation, other groups have found that antibodies directed against the CD11a/ CD18 integrin pair did inhibit the stimulation (Fischer et al., 1992). However, this could possibly be due to a requirement for integrin binding to bring the two cells close enough for the superantigen to cross link the MHC and TCR. Data, obtained during restimulation of the T cell blasts, indicated that superantigen presented by HLA-DR was sufficient for both proliferation and IL-2 production but that addition of B7 as a costimulatory molecule, whilst minimally affecting proliferation, considerably enhanced IL-2 production. This may suggest that superantigens are capable of providing some costimulatory signals (Damle et al., 1993) and in keeping with the observation that MHC-superantigen complexes can also stimulate proliferation in resting T cells (Sansom et al., 1993). This raises the question of which *in vitro* TCR stimulus provides the most physiological signals. It has become evident that the signals resulting from a peptide encounter do not equate with those induced by superantigen binding nor those arising from αCD3 crosslinking or stimulation of the cell with PMA and ionomycin. Data obtained from CD28 knockout mice indicate that in the absence of CD28 it is possible to initiate but not sustain an immune response. Thus stimuli such as αCD3 which require costimulation to initiate a response may not be fully activating the TCR. My data, in agreement with other reports, has shown that whilst superantigen stimulation alone appears adequate to initiate activation in these cells, further costimulation may be required for sufficient IL-2 production and long term survival of the culture. Thus superantigen stimulation which ligates the αβ chains of the TCR may be more physiologically relevant than crosslinking αCD3 and whilst this SEA T cell blast model does not appear to respond to αCD3 stimulation exactly like a resting T cell the latter

may not actually be a physiologically relevant response. Furthermore there are several advantages of this model compared to resting cells and the frequently used T cell model, Jurkat cells. The T cell blasts have a normal phenotype, are not transformed and undergo normal activation and reactivation cycles which are dependent upon IL-2 and costimulatory signals. Thus they are normal cycling, activated T cells and repond as such. Furthermore the cells can be quiesced to reduce their basal responses and then activated by B7 stimulation alone to enable the observation of the costimulatory signals in isolation, independently of TCR derived signals.

In this study it was demonstrated that B7 stimulation of quiescent SEA blasts activated both the PI3 Kinase and acidic sphingomyelinase signalling pathways and induced AP-1 and NFκB transcription factor complexes. Since these effects were not observed in resting cells, this indicates that the B7-induced responses may be due to a synergy between the CD28-mediated signals and other signals generated during the full activation of the cells 8-10 days previous. This observation is in agreement with other reports which state that there can be a delay between the first and second signals which will still result in full activation of the cells (Vandenburghe et al., 1992; Lu et al., 1992;) and is further supported by the observation that the B7 response is time dependent. However, due to the lack of NFAT and IL-2 production this implies that a fully activated TCR pathway is not a characteristic of these cells and the identity of the signal synergising with the B7 induced signal still needs further investigation. Data obtained from the enzyme inhibitor studies indicated that both PKC and and an active calcium pathway were involved in the B7 mediated responses. However, since CD28 signalling is proposed to be cyclosporin A insensitive (June et al., 1987) it is possible that a preactivated calcium pathway may be involved.

The observation that B7 could partially abrogate the cyclosporin-induced inhibition of PMA/ ionomycin stimulated proliferation and IL-2 production in the T cell blasts is somewhat surprising and indicates that either in the presence of PMA and B7 calcineurin is not required for T cell function or that the signals from the activated PKC enzyme enable the synergy of the TCR and costimulatory pathways to occur downstream of calcineurin. More information on the pathways involved may be gained from the assessment of NFAT induction in such a situation since this has been shown to be dependent upon the activation of calcineurin and hence its downstream effects.

The role of PKC is a little more difficult to decipher since the existence of various isotypes each having varying sensitivities to PMA and calcium and different positive and negative regulatory targets (Hug and Sarre, 1993) makes the delineation of the pathways extremely complex. Previous observations made following the activation of Jurkat cells with PMA has only served to confuse the issue since I have now shown that the

responses induced by this agent are completely different in Jurkats compared to T cell blasts and resting T cells. Currently without the aid of specific isotype activators and inhibitors it is not possible to distinguish TCR-activated PKC from that regulated by other signalling pathways. In future studies it may be possible to investigate the role of PKC using Jurkat cells with dominant negative or constitutively activated PKC isotypes.

The response to the B7 stimulation of quiesced SEA blasts was an induction of proliferation but not of detectable IL-2 production. This appears to indicate discordancy of proliferation and IL-2 production and may imply that either they are regulated by two different signalling pathways or that whilst for proliferation the CD28 signal is limiting in this model, IL-2 production requires concomitant activation of both the TCR and CD28 pathways. Whether or not either proves to be correct, one possibility is that the observed proliferation may be being driven by another cytokine other than IL-2. Although it is currently accepted that IL-2 is the main T cell proliferative cytokine other cytokines such as IL-4, IL-6 and IL-7 can induce proliferation but appear to affect only specific subsets, however, it is possible that the cytokine output from T cells changes as activation occurs. Since such a stimulation did not induce NFAT it is perhaps not surprising that no IL-2 was observed and may thus point to the production of a non-NFAT regulated cytokine.

Previous reports of downstream targets of CD28 signalling have largely concentrated on its ability to increase gene transcription by mechanisms of mRNA stabilisation (Lindsten et al., 1989a) and induction of a specific regulatory transcription factor CD28RC. First identified by Fraser and Weiss (1991) in PMA/ ionomycin/ αCD28 stimulated Jurkat cells, induction of this factor has since been shown to be independent of CD28 receptor ligation although this does not preclude activation of the costimulatory pathway by the high dose of PMA. The characteristics of the CD28RC indicate it is mainly composed of the NFκB like Rel family members (Ghosh et al., 1993) although this is currently disputed by another group (Verweij, personal communication). This, in combination with reports that CD28 ligation could increase translocation of c-rel (Bryan et al., 1994) induced me to study the effects of CD28 ligation on transcription factor induction in my SEA blast T cell model. Since these cells could be induced to produce IL-2 when stimulated with DR4-SEA and DR4/B7-SEA but not with B7 alone, a comparison of IL-2 gene promoter transcription factor induction was made in these stimulated cells. Interestingly whilst the DR4-SEA and DR4/ B7-SEA stimulations induced all three transcription factor complexes studied, NFAT, NFκB and AP-1, B7 stimulation alone was found to upregulate NFκB and AP-1 but not NFAT.

The induction of NFκB following B7 stimulation of the resting SEA blasts indicates that this signal is sufficient to release the NFκB complex from its inhibitory protein IκB.

Although previous reports have shown the ability of CD28 derived signals to increase c-rel translocation in the presence of PMA the effects of CD28 stimulation alone were not investigated. This may indicate that in these blasts the non-CD28, PMA-regulated pathway is still active and that the signals originating from the B7 stimulation synergise with this to induce an NFκB DNA binding complex. My data has shown this induction to be mediated via activation of acidic sphingomyelinase due to the inhibition of NFκB induction by chloroquine. Recently Cifone et al (1995) reported that acidic sphingomyelinase could be activated by DAG produced from the the action of PLC on the membrane lipid phosphorylcholine and since PMA is a synthetic form of DAG this too may activate the acidic sphingomyelinase pathway. Furthermore in chapter 3 I proposed that high concentrations of PMA may activate the costimulatory pathway thus this proposed interaction of DAG with acidic sphingomyelinase suggests a possible target for this activation. Previous reports have indicated that ceramide generated from sphingomyelin by the sphingomyelinase enzyme mediates an effect on NFκB via activation of PKCζ (Muller et al., 1995b; Lozano et al., 1994). However, as this isotype is supposedly insensitive to PMA this cannot be the control point for the PMA regulation of the pathway and further investigations will be required to determine the point of synergy of the two signals.

Similar conclusions may be drawn from the B7 stimulated induction of AP-1. This shows that CD28 signalling is linked to AP-1 production but does not indicate whether this occurs alone or in synergy with a second active signal in these cells. It has been shown that B7 stimulation of the blasts induced JNK activity. Whilst previous reports demonstrated that PMA or αCD28 alone were insufficient to fully activate JNK in combination full activation of the kinase was observed (Su et al., 1994). However, since JNK activation only regulates c-jun a second signal would also be required to activate c-fos in order to generate a complete AP-1 complex. Thus it appears in our model that the B7 stimulated signals arising from CD28 engagement are synergising with a previously activated pathway to induce AP-1 and NFκB activity. This highlights the importance of the costimulatory signal in the activation of T cells since it now appears that under physiological conditions in normal, untransformed cells, CD28 derived signals are required to synergistically activate the pathways leading to the induction of transcription factors which can then regulate gene expression. Since prior to this work the outcome of CD28 signals and the specific role they played in the activation of normal T cells was unknown, the discovery of AP-1 and NFκB induction is a considerable advancement. The failure of TCR-stimulated cells to induce IL-2 production can now be explained by a lack of functional transcription factors. Thus whilst TCR derived signals appear able to activate OCT and cause the translocation of the cytoplasmic subunit of NFAT, additional CD28-derived signals are required for the induction of NFκB and AP-1 which makes these crucial factors in the regulation of T cell activation. This has been previously

shown by the effects of glucocorticoids which suppress AP-1 and NFκB activity (Auphan et al., 1995; Scheinman et al., 1995) and which were deemed to be more specific than the general immunosuppressants which target the calcium regulated NFAT pathway. Thus inhibition of the costimulatory signals seems to be a highly effective way of regulating T cell activation.

The activation of both the PI3Kinase and acidic sphingomyelinase pathways was also demonstrated following B7 stimulation of these SEA blasts in agreement with previous reports (Prasad et al., 1994; Pages et al., 1994; Boucher et al., 1995). However, it is not yet known whether *in vivo* both pathways are activated simultaneously or whether the signalling outcome of CD28 ligation is dependent upon other factors such as the activation state of the cell. My data suggested that whilst PI3 Kinase activation could be demonstrated in these cells, inhibition of kinase activity did not appear to significantly affect proliferation. Furthermore previous studies have shown an increase in IL-2 output from Jurkat cells and T cell blasts preincubated with wortmannin (Ueda et al., 1995). This is consistent with a separation of the proliferative and IL-2 pathways in activated T cells and also indicates that PI3 Kinase may play a role as a negative regulator of IL-2 production. Very little is actually known concerning the downstream targets of the D-3 phosphoinoside molecules generated following PI3 Kinase activation although recently reports of PI3 kinase regulation of PKB (Burgering and Coffer, 1995), PKCζ () and rac/ cdc42 (Hawkins et al., 1995) have emerged. Therefore contrary to the above data this potentially means that PI3 kinase can also directly regulate transcription factor induction as PKCζ induces NFκB and rac is upstream of JNK.

Identification of the two transcription factor targets of CD28 signalling provided a convenient end point for the further assessment of the involvement of the PI3 Kinase and acidic sphingomyelinase signalling pathways. The results indicate that whilst AP-1 is probably regulated by both enzymes, NFκB induction is controlled by acidic sphingomyelinase but is independent of PI3 Kinase. The reason for the observed variability in chloroquine-mediated inhibition of AP-1 is not known but may be dependent upon the exact activation state of the PI3 Kinase pathway at the time of the experiment since this provides an alternative induction mechanism. In the absence of PI3 Kinase activation as implied by the relative insensitivity of the cells to wortmannin, AP-1 is presumably induced via the sphingomyelinase pathway alone. However, with only one pathway inducing AP-1 synthesis instead of two this may result in a decrease in the total amount of AP-1 to below the threshold required for IL-2 gene transcription. This may result in a shift of the cytokine output away from IL-2 to a second cytokine with a lower AP-1 threshold. This is just one possible explanation for the observed lack of IL-2 output from these cells and has yet to be substantiated. However, this theory implies that in

activated cells only the acidic sphingomyelinase pathway is necessary for IL-2 costimulation.

What then is the role played by PI3 Kinase as in the above discussion it appears that all the effects of CD28 can be mediated via acidic sphingomyelinase ? The answer may lie in the downstream targets of PI3 Kinase. Since PI3 Kinase has been shown to mediate the activation of the cell cycle regulator S6 kinase one role for PI3 Kinase may be to coordinate entry into the cell cycle. Resting T cells are known to lie outside the cell cycle in G_0 and require full stimulation to progress to G_1 whereas the T cell blasts probably only quiesce to G_1 which may explain their partially activated state and the apparent redundancy of PI3 Kinase in the activation of these T cells. However, PI3 Kinase may still have a role in the continued survival of activated T cells.

PI3 Kinase has always been considered as a pro-proliferative signal (Yao and Cooper, 1995) and the sphingomyelinase product, ceramide maybe a mediator of apoptosis (reviewed by Pushkareva et al., 1995) so it is rather paradoxical that they should both be activated by a surface molecule whose major function is to stimulate proliferation. However, since activation of the acidic sphingomyelinase pathway alone is thought to induce apoptosis the role of PI3 Kinase in activated cells may be in the provision of synergistic signals to prevent apoptosis and promote cell survival via activation of PKB and S6 kinase. This suggests that even in activated cells both signalling pathways are required. In agreement with this are reports that B7-1 is capable of activating both pathways although data concerning the effects of B7-2 are awaited. However, since currently no great differences in stimulatory capabilities have been identified using the two different ligands and that recently Cai et al. (1995) have demonstrated B7-2 association with PI3 Kinase it is likely that B7-2 may also activate both signalling pathways.

Interestingly, previous work has proposed CTLA-4 as a potent inducer of apoptosis (Gribben et al., 1995) supported by the observation that CTLA-4 knockout mice show extreme lymphoproliferative disorders (Waterhouse et al., 1995; Tivol et al., 1995) and possibly indicating activation of the sphingomyelinase pathway. However CTLA-4 has also been shown to interact with PI3 Kinase (Schneider et al., 1995) although this does not necessarily mean the pathway is active and the binding may be inhibitory. Thus the lack of inhibitory effect of wortmannin on proliferation and IL-2 production in activated cells does not exclude a role for PI3 Kinase in cell survival and indicates that different emphases may be placed on different signalling pathways dependent upon the activation state of the cell and the surface molecules involved in the activation.

There are still many unanswered questions and further studies to investigate the interaction of the two CD28 mediated signalling pathways are obviously required. This work has shown that superantigen driven T cell blasts are a useful model for the investigation of these signalling pathways and may prove to be of further use yet in studies investigating the intracellular events associated with anergy and the CTLA-4 mediated apoptotic effects.

CHAPTER 6.0 - CONCLUSIONS

The aim of this work was to investigate the role of CD28 mediated signals in the activation of T lymphocytes with specific reference to the regulation of IL-2 gene transcription. This has been undertaken using a number of different techniques and cell types and has established several important points.

Whilst in resting cells the activation of CD28 is necessary to drive proliferation and IL-2 production, in activated T cells CD28 signals alone can drive proliferation but do not result in the generation of IL-2, implying a separation of the two activation pathways. This questions the role of IL-2 as the main T cell proliferative cytokine in these cells and indicates there may be a change in the nature of the cytokine output from the cells after activation but this will require further investigation. One hypothesis derived from this is that whilst in resting T cells CD28 is required to generate IL-2 to launch the cells into the cell cycle, once activated, IL-2 is no longer required by the cells to maintain the activated state.

Although a number of papers have been published concerning the role of CD28 in T cell activation the majority of the previous work has been carried out in mitogenically activated transformed cell lines such as PMA stimulated Jurkat cells. However, this current work has shown that transformed cell lines such as Jurkat cells show substantially different responses to those of normal T cells. In addition to their constitutive, IL-2 independent proliferation, stimuli such as PMA which can induce proliferation and IL-2 output in normal T cells have been shown to inhibit the proliferation of Jurkat cells whilst stimulating IL-2 output. This provides further evidence for the divergence of proliferation and IL-2 production in activated cells and indicates that in Jurkat cells the proliferative pathway is inversely regulated by PMA.

Due to the large number of PKC isotypes and possible target sites, PMA can no longer be considered a useful and informative T cell activation stimulus. Contrary to just providing TCR related PKC signals, PMA now appears to have targets on a number of signalling pathways and the high doses of PMA normally used in activation experiments appear to result in the activation of several of these signalling pathways simultaneously. Thus the observed responses are the summation of the activation of multiple signalling pathways. The discovery that the specificity of the compound could be increased by reducing the concentration of PMA has improved its usefulness although further studies to identify the target sites are required.

This project has involved the development of an alternative model system for the investigation of the costimulatory pathway. The use of superantigen driven T cell blasts

avoids many of the complications associated with the use of constitutively activated transformed cells whilst being more readily available than large numbers of resting T cells. The T cell blasts are a good physiological model of resting T cells in that they require two signals for full activation at the beginning and end of their cycle whilst during active cycling (day 4-12) they can be induced to proliferate in response to a single stimulus. However, this does not result in IL-2 production indicating that IL-2 transcription is more tightly regulated and may require simultaneous activation of both the TCR and costimulatory signalling pathways. This correlates with the transcription factor production whereby in these activated cells CD28 signals can generate AP-1 and NFκB whilst additional TCR signals are required for the induction of NFAT.

This work confirmed that CD28 utilises both the PI3 Kinase and acidic sphingomyelinase signalling pathways although the present data suggests these two pathways may have different roles. Although PI3 Kinase was shown to regulate AP-1 generation, the inhibition of this enzyme did not have a major effect on the proliferation of the T cell blasts whilst it did inhibit resting T cell function. I propose that the main role for PI3 Kinase may be in promoting the entry of resting cells into the cell cycle probably via activation of S6 kinase but that in activated cycling cells PI3 Kinase recruitment is not an absolute requirement. Interestingly whilst wortmannin had very little effect on the T cell blasts they were effectively inhibited by rapamycin thus in cycling cells a constitutive activation of the pathway between PI3 Kinase and TOR appears to occur. Since currently this position is proposed to be occupied by PKB this could prove to be a very important mediator of cell cycle and survival in activated cells.

Activation of acidic sphingomyelinase resulted in the generation of both NFκB and AP-1 implying that this pathway may be important in the regulation of IL-2. This at first appears contradictory to the observations that other receptors, such as TNF and Fas, which activate this pathway are possible mediators of apoptosis. However, since the activation of acidic sphingomyelinase by CD28 occurs in parallel to an active PI3 Kinase " survival" pathway and usually occurs in the presence of a TCR signal the reconciliation of these data is likely to involve the relative balance between different signalling pathways and the synergism of activation signals which override the apoptotic mechanism.

This work has provided some new and interesting information concerning the possible roles for the different CD28-associated signalling pathways and has identified NFκB and AP-1 as two downstream targets of CD28 derived signals. However, even from these initial studies the complexity of the interacting pathways which mediate the activation process is evident. It is hoped that as improved T cell models are developed and our knowledge and understanding of the processes involved in T cell activation increase, the

complexities will be resolved and rational manipulation of the immune system will be possible.

CHAPTER 7.0 - FUTURE WORK

The work carried out represented a starting point for studies into the signalling mechanisms associated with CD28-mediated transcriptional activation and has identified a number of important questions which can now be addressed in future work.

1) If the use of mitogenically activated Jurkat cells is to be continued in studies of T cell activation it will be necessary to develop a greater understanding of the effects of PMA and the activation requirements and regulation of the intracellular signalling pathways of the Jurkat cells. Current data indicate that PMA may activate both the TCR -related MAPK pathway and the costimulatory regulated acidic sphingomyelinase pathway although there appears to be a difference in PMA sensitivity of the two pathways as shown by the differences in cell responses using high (both) and low (MAPK only) dose PMA. Although the involvement of PKC enzymes in TCR signalling is well known further investigations will be required to assess the involvement of PMA in the activation of the sphingomyelinase enzyme and direct activation of the downstream targets of ceramide such as NFκB and CAPK. Furthermore data acquired in the presence of the enzyme inhibitor chloroquine may provide further information on the sites of action of PMA.

2) Data acquired from Jurkat cell proliferation assays and the FACS analysis of cell cycle indicated that incubation of these cells with PMA resulted in cell death and that this was more significant at low dose PMA (ie activation of the TCR pathway in the absence of costimulatory pathway activation). Furthermore addition of B7 transfectants to PMA treated Jurkat cells did appear to partially restore their ability to proliferate indicating that activation of the costimulatory pathway may have a protective effect against apoptosis. FACS analysis of Jurkat cell cycle using a dose range of PMA in the presence and absence of B7 stimulation should therefore be carried out to further investigate this protective effect.

3) Further development of this project should involve a more in depth study of the relative roles of PI3 Kinase and acidic sphingomyelinase pathways in both T cell survival and in the induction of anergy and apoptosis. Rather than being distinct events it appears likely that a complex interplay of the various signalling pathways controls the final outcome of the individual T cell in response to a stimulus. A comparison of CD28 and CTLA-4 mediated intracellular signals may thus shed some light on the important regulatory molecules which control these outcomes and may help in our understanding of the regulation of T cell activation. In addition a comparison of the intracellular downstream signalling events induced by B7-2 as opposed to those observed with B7-1

may provide further information on the roles of these molecules during the progression of T cell activation.

4) The failure to detect IL-2 production from the stimulated T cell blasts indicated that the B7 stimulus was insufficient to induce gene transcription and correlated with a lack of NFAT induction. However, at earlier time points (day 6) when the cells demonstrated high levels of basal proliferation and when gel shift assays revealed significant levels of all three transcription factors investigated it was still not possible to detect any IL-2 output. However, at this time the cells may be producing minimal levels of IL-2, undetectable in the supernatant, which are just sufficient to maintain autocrine proliferation and this should be investigated using RT PCR analysis to detect the presence of IL-2 mRNA in these cells.

5) Previous studies of T cell activation have involved the use of many different stimuli to activate the TCR and current data now indicate that these may not be equivalent. Transcription factor analysis revealed that stimulation of the T cell blasts with DR4-SEA induced NFAT, AP-1 and NFκB transcription factors as did PMA and ionomycin implying an activation of both the TCR and costimulatory pathways. An interesting comparison would thus be the effects of αCD3 stimulation on transcription factor induction since this is not thought to activate the costimulatory pathway.

6) The observation that B7 could costimulate IL-2 production in PMA and ionomycin treated T cell blasts in the presence of cyclosporin A implied an interaction with or a bypass of the calcium/ calcineurin pathway although the mechanism of this is unknown. However, as the cytoplasmic component of NFAT is a specific downstream target of the calcium/ calcineurin pathway, the assessment of NFAT induction in these treated cells could provide useful information on the interaction of the TCR and costimulatory signalling pathways.

Unfortunately there was insufficient time to investigate these questions but as the assays have been established and the SEA T cell blasts shown to be a good physiological model it is hoped that this work may be developed further.

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APPENDIX 1 - CELL CULTURE MEDIA

1) R10 for T cell blasts and purified peripheral blood T cells

1x RPMI 1640	500mls(Life Technologies Cat. No.31870-025)
10% FCS	50mls (Life Technologies Cat. No.10108-074)
100U/ml Penicillin	5mls of 10000U/ml / 10mg/ml stock
0.1mg/ml Streptomycin	(Life Technologies Cat. No.15140-114)
2mM Glutamine	5mls of 200mM stock
	(Life Technologies (Cat No.25030-024)

2) R10 for Jurkat cells

1x RPMI 1640	50mls of 10x stock (Life Tech. Cat. No.22511-026)
10% FCS	50mls
100U/ml Penicillin	5mls of 10000U/ml / 10mg/ml stock
0.1mg/ml Streptomycin	
0.2% Sodium bicarbonate	15mls of 7.5% stock (Life Tech.Cat. No.25080-060)
0.015N Sodium hydroxide	0.8mls of 10N stock
2mM Glutamine	5mls of 200mM stock
	(Life Technologies (Cat No.25030-024)
Milli Q H_2O	400mls

3) Glutamine- Free media for CHO transfectants

1x DMEM	55mls of 10x stock (Life Tech. Cat. No.12501-029)
10% FCS	55mls
100U/ml Penicillin	5mls of 10000U/ml / 10mg/ml stock
0.1mg/ml Streptomycin	
0.4% Sodium bicarbonate	28mls of 7.5% stock
1mM Sodium pyruvate	5mls of 100mM stock (Life Tech. Cat. No.11360-039)
1x Nucleosides (see below)	5mls of 100x stock
Milli-Q H_2O	400mls

4) Nucleosides

These were dissolved in 100mls of Milli-Q H_2O and filter sterilised.

Adenosine	70mg	Uridine	70mg
Cytidine	70mg	Thymidine	24 mg
Guanosine	70mg		

APPENDIX 2 - BUFFER SOLUTIONS

1) TRIS-EDTA (TE) pH 8.0 :-

10x	0.4M Tris Base	48.4g
	0.01M EDTA	3.72g
	H_2O	to 1L

2) Tris-Borate-EDTA (TBE) pH 7.8 :-

10x	0.445M Tris Base	54g
	0.445M Boric Acid	27.5g
	0.01M EDTA	3.72g
	H_2O	to 1L

3) Tris Acetate EDTA (TAE) pH 8.0 :-

10x	0.4M Tris Base	48.44g
	0.05M NaAc	4.1g
	0.01M EDTA	3.72g
	H_2O	to 1L

4) SDS Gel Running Buffer (TSG) pH 8.3 :-

1x	25mM Tris Base	3.03g
	192mM Glycine	14.42g
	0.1% $^{w}/_{v}$ SDS	1g
	H_2O	to 1L

5) Loading Buffer :-

6x	40% $^{w}/_{v}$ sucrose	4g
	0.25% $^{w}/_{v}$ Bromophenol Blue	25mg
	H_2O	to 10mls

6) Sample Buffer pH 6.8 :-

1x	100mM Tris HCl	0.12g
	1% $^{w}/_{v}$SDS	0.1g
	0.2% $^{w}/_{v}$Bromophenol Blue	0.02g
	20% $^{v}/_{v}$glycerol	2mls
	2.5% $^{v}/_{v}$β-Mercaptoethanol	0.25g
	H_2O	to 10mls

APPENDIX 3 - GELS

1) 5% Non- Denaturing Polyacrylamide Gel :-

0.5x TBE buffer	1.5mls of 10x stock
5% acrylamide	5mls of 30% 37.5:1 acrylamide:bis stock
0.1% Ammonium Persulphate	300µl of 10% stock
0.01% TEMED	30µl (Sigma T8133)
H_2O	to 30mls

2) 10% SDS-PAGE Gel :-

0.375M Tris-HCl	8.0mls of 1.5M stock pH 8.8
10% Acrylamide	10.6mls of 30% 37.5:1 acrylamide: bis stock
0.1% $^{W}/_{V}$ SDS	0.32mls of 10% $^{W}/_{V}$ stock
0.03% Ammonium Persulphate	120µl of 10% stock
0.01% TEMED	30µl
H_2O	to 32mls

3) 1% Agarose gel :-

1% agarose	1.5g
1x TAE	150mls
0.005% $^{V}/_{V}$ Ethidium Bromide	7.5µl

4) 6% Sequencing Gel :-

7M Urea	63g
1x TBE	15mls of 10x stock
6% Acrylamide	30mls of 30% 19:1 bis: acrylamide stock
0.05% Ammonium Persulphate	800µl of 10% stock
0.005% TEMED	80µl
H_2O	to 150mls

APPENDIX 4 - BACTERIA CULTURE

All media prepared was autoclaved and then cooled prior to use.

1) General Broth :-

Tryptan	10g
Yeast Extract	5g
Sodium Chloride	10g
H_2O	to 1L

An alternative broth was prepared using Circle Gro (3.4%$^{w}/_{v}$) for cultures requiring a richer medium.

2) Agar plates :-

3.4%$^{w}/_{v}$ Circle Gro	3.4g
2%$^{w}/_{v}$ Agar	2g
H_2O	to 100mls

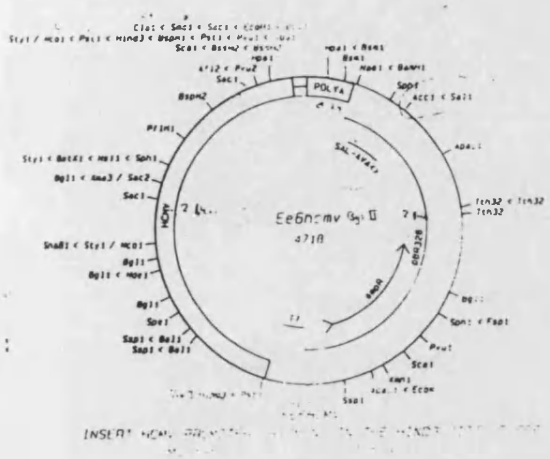
Ampicillin (50µg/ml) was added after autoclaving before the plates were poured.

3) SOC media :-

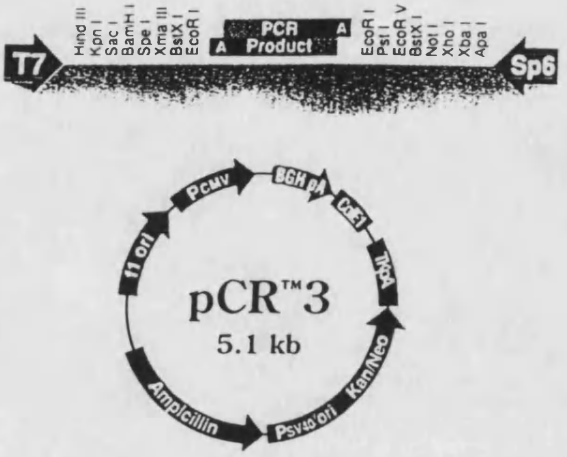
Tryptan	20g
Yeast Extract	5g
Sodium Chloride	0.5g
20mM Glucose	
H_2O	to 1L

APPENDIX 5- PLASMID VECTORS AND CONSTRUCTS

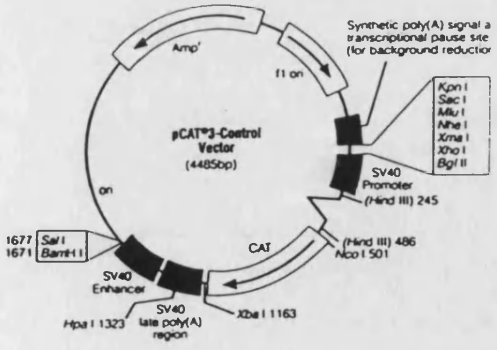
1) pEe6 Expression Vector used for transfection of surface antigens CHO cell hosts.



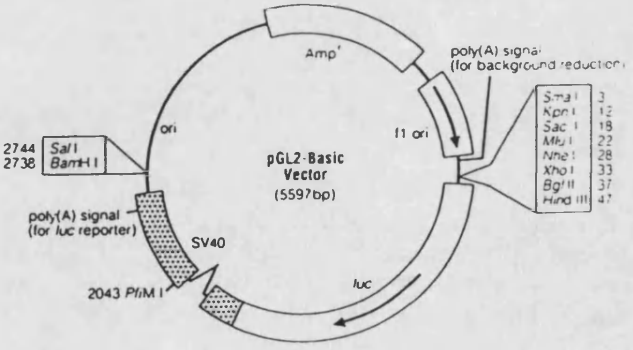
2) pCR3 Expression Vector used the for the subcloning of the IL-2 into promoter to generate the footprint probe.



3) pCAT Control Vector used in the transfection of Jurkat cells to obtain constitutive CAT enzyme activity.



4) pGL2 Basic Vector into which were inserted multiple transcription factor binding sites to regulate the luciferase enzyme activity.



PRESENTATIONS AND PUBLICATIONS

The work carried out during my PhD has resulted in a number of publications which are listed below in addition to an oral presentation at the 12th European Immunology Meeting in Barcelona in June 1994 entitled ' CD28 signalling in T lymphocytes'.

Abstracts

Edmead CE, Ward SG, Hall ND and Sansom DM. CD28 Signalling in T Lymphocytes and the role of the CD28RC. 12th European Immunology Meeting. Barcelona. June 1994.

Papers

Sansom DM, Edmead CE, Hall ND, Westwick J and Ward SG. Signalling via CD28 involves association with and activation of phosphoinositide 3 kinase.(1994). J. Cell. Biochem. 433

Sansom DM, Edmead CE, Parry R, Wilson A and Ward SG. The T cell costimulatory molecule CD28 couples to multiple signalling pathways. In ' Lymphocyte Signalling' : Eds - Harnett M, Rigley K and J Wiley and Sons. In press.

Edmead CE, Patel YI, Wilson A, Boulougouris G, Hall ND, Ward SG and Sansom DM. Induction of AP-1 and NFκB by CD28 stimulation involves both PI3 Kinase and Acidic Sphingomyelinase signals. Manuscript in preparation.